Investigation of *Salmonella* prevalence, quantification with a method comparison, and serotyping in market hog lymphoid tissues

by

Erin Elizabeth Fashenpour

B.S., Colorado State University, 2021

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science
Animal Sciences & Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

Major Professor
Sara E. Gragg, Ph.D.

Copyright

Abstract

The United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) will soon release performance standards for *Salmonella* in pork that will affect change in the meat industry. Although somewhat limited in comparison to other protein sources, food safety professionals need to address foodborne illness caused by pork-related products. Lymph nodes (LNs) have been researched frequently in recent years in the meat industry to investigate *Salmonella* harborage and presence in ground product. The first study evaluated the prevalence and concentration of *Salmonella* in market hog LNs and tonsils. Regional and seasonal variability were also explored. An enumeration method comparison was performed between the 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®. Samples were collected from six participating pork processing facilities, located in three pre-determined regions (east, central, and west). Samples were collected during three seasons (winter, spring, and fall) from each plant, where 30-35 carcasses were selected at random on the harvest floor. From each carcass, seven sample types were collected, including mesenteric, subiliac, superficial inguinal, prescapular, axillary, tracheobronchial LNs, and tonsils. Samples were evaluated using BAX *Salmonella* Real-time PCR Assay for a *Salmonella* detection step. Positive samples were then evaluated for *Salmonella* quantification using BAX®-System-SalQuant® methods. Of the 4,132 samples collected, 14% were positive for *Salmonella*. A season-by-region interaction was observed for *Salmonella* prevalence in axillary and tracheobronchial LNs, and tonsils ($P<0.05$). Of these three sample types, tonsils had the highest prevalence observed in the eastern region in the spring season (74.2%). In general, *Salmonella* prevalence appears to be greatest in the eastern region during spring, though this varies with sample type. Tonsils and mesenteric LNs had the highest overall prevalence at 35% and 34%, respectively, however a season-by-region interaction

was not detected for mesenteric LN prevalence. *Salmonella* prevalence by carcass was 62% out of the 601 total carcasses; a season-by-region interaction was observed (P=0.001). The highest carcass prevalence occurred during the spring in the eastern region (90.9%), and the lowest prevalence occurred during the spring in the central region (26.0%). Only tonsils and mesenteric LNs were statistically evaluated for *Salmonella* concentration. Median *Salmonella* concentrations fell below the limit of quantification for mesenteric LNs, while median concentration for tonsils was 2.18 $\log_{10}$ CFU/sample using BAX®-System-SalQuant®. The method comparison results showed that the 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® can be used for quantification, however there are benefits and pitfalls to both. Following statistical analysis, a kappa coefficient value of 0.384 was observed between 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®, indicating a fair agreement between the *Salmonella* quantification methods. A t-test was also performed on the 83 samples that were quantified by both methodologies, where no significant difference was observed in the mean log CFU/sample (P>0.05). *Salmonella* was isolated from market hog LNs and tonsils, and isolates were subjected to serotyping. Serotype is an important variable to consider when evaluating the risk of contamination from these sample types. Not all serotypes are pathogenic to humans, however some are known to be more virulent or even deadly. The most isolated serotypes included monophasic *S.* Typhimurium, followed by *S.* Agona and *S.* Schwarzengrund, all of which can be pathogenic to humans. While most isolates were recovered from mesenteric LNs and tonsils, pathogenic *Salmonella* was recovered from peripheral LNs, which pose a greater risk of contamination in consumer products. These data will allow for a risk assessment of *Salmonella* prevalence, concentration, and serotypes to

improve understanding of how contaminated market hog lymph nodes affect risk to human health.

Table of Contents

List of Figures viii
List of Tables x
Acknowledgements xi
Chapter 1 - Introduction and research questions 1
Research questions 3
References 4
Chapter 2 - Literature Review 7
2.1 *Salmonella* background information 7
Salmonella nomenclature 9
Salmonella serotype 9
Salmonella pathogenicity and methods of infection 12
Public health and economic impact of *Salmonella* 15
Salmonella in swine production 16
Pork processing 17
Salmonella in pork lymph nodes 19
2.2 Overview of the lymph system 21
2.3 Overview of PCR 22
BAX quantification from PCR 23
2.4 Government standards for *Salmonella* (USDA) 26
References 29
Chapter 3 - Comparison of *Salmonella* Enumeration Methods and an Investigation of *Salmonella* Prevalence and Quantification in Market Hog Lymph Nodes and Tonsils in Several Regions and Seasons of the United States 41
Abstract 41
3.1 Introduction 43
3.2 Materials and methods 45
3.2.1 Sample collection 45
3.2.2 Sample processing 46
3.2.3 *Salmonella* enumeration 47

3.2.4 *Salmonella* detection 48
3.2.5 *Salmonella* confirmation and isolation 48
3.2.6 Data processing 49
3.2.7 Statistical analysis for prevalence, enumeration, and method comparison 50
3.3 Results and discussion 53
3.3.1 Prevalence and enumeration 53
3.3.2 Method comparison 67
3.4 Conclusions 74
References 76
Chapter 4 - Serotyping of *Salmonella* Isolated from Market Hog Lymph Nodes and Tonsils 82
Abstract 82
4.1 Introduction 82
4.2 Materials and methods 84
4.2.1 Sample collection 84
4.2.2 Sample processing 85
4.2.3 Serotyping 85
4.3 Results and discussion 85
4.5 Conclusions 94
References 96
Chapter 5 - Summary and Conclusions 104
5.1 *Salmonella* in market hog lymph nodes 104
5.2 Suggested further research 105
References 107

List of Figures

Figure 3-1. Graphical representation of *Salmonella* prevalence (%) by sample type with corresponding p-values where the season-by-region interaction was significant for prevalence (axillary LNs (*P*=0.038), tonsils (*P*=0.006), and tracheobronchial LNs (P=0.007)). The figure is divided by (A) *Salmonella* prevalence by region (central, east, and west) and (B) *Salmonella* prevalence by season (winter, spring, and fall) for the three significant (*P*<0.05) sample types..... 55

Figure 3-2. Graphical representation of *Salmonella* prevalence (%) by sample type with corresponding p-values where the season-by-region interaction was not significant for prevalence (mesenteric LNs (*P*=0.188), pre-scapular LNs (*P*=0.157), subiliac LNs (*P*=0.136), and superficial inguinal LNs (*P*=0.122)). The figure is divided by (A) *Salmonella* prevalence by season (winter, spring, and fall) for each sample type and (B) *Salmonella* prevalence by region for each sample type (central, east, and west) for the four sample types that were not significant (*P*>0.05) for season-by-region interaction. 56

Figure 3-3. Model-based estimate of the distribution of the number of *Salmonella* positive sample types by season and region. 63

Figure 3-4. Bar charts of *Salmonella* $\log_{10}$ (CFU/Sample) enumeration from ANOVA test organized by region with season for sample types where season-by-region interaction was significant (mesenteric LNs (*P*=0.014) and tonsils (*P*=0.031) and were *Salmonella* positive. Only mesenteric LNs and tonsils were statistically analyzed for *Salmonella* enumeration using BAX®-System-SalQuant®. 65

Figure 3-5. Graphical representation of the comparison between 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® for enumeration of *Salmonella* in pork lymph nodes. A) Enumeration of *Salmonella* (Log CFU/Sample) in pork lymph nodes using 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® (*n* = 378 samples). The dots represent the actual data points. LOQ: Limit of Quantification. B) Raincloud plot of *Salmonella* counts (Log CFU/Sample) using 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® in pork lymph nodes with counts above limit of quantification for both methodologies (*n* = 83 samples). In each boxplot, the horizontal line crossing the box represents the median, the bottom and top box are the lower and upper

quartiles, the vertical top line represents 1.5 times the interquartile range, and the vertical bottom line represents 1.5 times the lower interquartile range. The dots represent the actual data points. Distribution plots were generated using the kernel density estimates as the probability density function of a continuous variable. 69
Figure 4-1. Graphical representation of *Salmonella* serotypes isolated from market hog mesenteric LNs organized by isolate count. 88
Figure 4-2. Graphical representation of *Salmonella* serotypes isolated from market hog tonsils organized by isolate count. 89
Figure 4-3. Graphical representation of *Salmonella* serotypes isolated from peripheral market hog LNs (subiliac, superficial inguinal, pre-scapular, and axillary LNs) organized by isolate count. 90

List of Tables

Table 3-1. Explanatory variables in a model representing the level of *Salmonella* contamination in a sample (detected and quantified by BAX®-System-SalQuant® were categorized into three groups: ND; [LOD, LOQ); ≥LOQ. 50

Table 3-2. Summary of *Salmonella* prevalence status reported by carcass in each season and region. Season by region interaction for *Salmonella* prevalence was significant for carcass (P=0.001). 58

Table 3-3. Distribution of *Salmonella* in lymph nodes by market hog carcass. Tonsils are included in the lymph node count. 62

Table 3-4. Contingency table of the number of *Salmonella* positive samples quantified from market hog LNs using both methodologies of 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®. 68

Table 4-1. *Salmonella* serotypes isolated, serotype frequency (by isolate and sample), and serotype prevalence (%) from market hog LNs and tonsils. 87

Acknowledgements

I want to thank my advisor, Dr. Sara Gragg, for allowing me to be one of her graduate students and entrusting me with an important research project. I would not have been able to complete my research without her guidance and support. Also, I want to thank my committee members Dr. Marcos Sanchez-Plata and Dr. Valentina Trinetta for their support in both my project and writing.

Next, I need to thank the graduate and undergraduate students in the K-State Food Safety and Defense Laboratory, including Kellen Habib, Jimeng Bai, Kellen Marcum, Zilfa Irakoze, Sirui Zhang, Alejandra Abrego, William Jaggers, Lauren Dornbusch, Mackenzie McAtee, and Grace Huynh. I truly would not have been able to complete my sample collection and laboratory work without their constant assistance. Also, a big thank you to Dr. David Vargas for his continued collaboration with the pork lymph node project.

Finally, I would like to thank my family and friends; my dad, Doug Fashenpour, for supporting me in my education and life decisions; my mom and sisters, Angie Fashenpour, Emily Fashenpour, and Dana Faist for always being my support group; and lastly, my dog, Moose, for the daily emotional support. I would not have been able to finish my master's degree without the help and encouragement of the people in my life.

Chapter 1 - Introduction and research questions

Salmonella is one of the most common sources of foodborne illness worldwide and is a critical public health concern for food safety, as millions of people are vulnerable to infection each year (CDC, 2019). According to the Centers for Disease Control and Prevention (CDC), it is estimated that 1.35 million people will become sick from a *Salmonella* infection, with 26,500 people becoming hospitalized and 420 people dying from these infections (CDC, 2023). The reservoirs for *Salmonella* are humans and production animals, such as cattle, hogs, goats, sheep, and poultry (Gut et al., 2018). *Salmonella* infection can vary in severity depending on the host factors, virulence factors, and serotype ingested; disease can range from gastroenteritis, septicemia, and enteric fever (Giannella, 1996).

When considering the impact the pork industry has on global food systems, it is important to address *Salmonella* contamination from pork products. Pork is the most consumed type of meat protein in the world (USDA, 2023). Specific to pork products, there were 72 *Salmonella* outbreaks between 1998 and 2015 that resulted in 2,215 illnesses (Self et al., 2017). More and more research has been conducted on lymph nodes (LNs) and their contribution to foodborne disease. Lymph nodes are found in the fatty tissue in the carcass, allowing them to evade normal pathogen reduction processes that occur during harvest (Gragg et al., 2013). These lymph tissues may also be found in trim products and be converted into ground pork (Zhang et al., 2019). Ground pork has a higher risk of containing pathogens such as *Salmonella* than whole cuts because of the combination of products from many carcasses (Zhang et al., 2019). Once the LN is opened by slicing or grinding, then *Salmonella* cells can cause cross contamination of the carcass or ground products (Chaves et al., 2017).

There are more than 2,500 serotypes of *Salmonella* that have been identified (Gut et al., 2018). *Salmonella* can be categorized as typhoidal or non-typhoidal (NTS); typhoidal strains cause enteric fevers, while NTS strains can cause gastroenteritis, and are the most commonly occurring type of *Salmonella* associated with agricultural products that cause human illness (Acheson & Hohmann, 2001; Gal-Mor et al., 2014). Serotypes are distinguished by antigens on the bacterial cell: the O, H and Vi antigens (Giannella, 1996). The O-antigen is located on the outside of the cell and is associated with the lipopolysaccharide layer (CDC, 2022; Giannella, 1996; McQuiston et al., 2011). The H-antigen refers to the flagella that allows the cells to move and can express in phase 1 or phase 2 (CDC, 2022; Giannella, 1996; McQuiston et al., 2011). The Vi antigen is a less frequently occurring antigen and is only found in a few serotypes; this antigen is located on the outside of the lipopolysaccharide layer that aids with *Salmonella* pathogenicity (Giannella, 1996; Wain et al., 2005). Serotyping is useful in understanding which strains of *Salmonella* are pathogenic to humans; it is also useful in outbreak tracing associated with foodborne illnesses (CDC, 2022). Of the more than 2,500 serotypes of *Salmonella*, only around 100 strains can cause illness in humans (Bonilla, 2021; CDC, 2023). There are host adapted strains that only cause illness in certain species. Some examples of these are *S.* Typhi which infect humans, *S.* Dublin that infect cattle, and *S.* Choleraesuis that infect hogs (Gut et al., 2018).

These studies aim to explore the prevalence of *Salmonella* in market hog LNs to evaluate which sample types contain this pathogen more frequently. Another objective was the enumeration of *Salmonella* from LNs contaminated with the pathogen to evaluate the dosage and subsequent risk to food safety. *Salmonella* isolates recovered from samples were serotyped to determine serotype distribution and how often the serotypes that cause human illness are present

in LNs. A method comparison between 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® method is included in this thesis to evaluate the effectiveness of the quantification methods used for market hog LNs.

Research questions

1. Is there a type of market hog lymph node or sample type that is consistently positive for *Salmonella*?
2. Is season, region, or the interaction of season and region significantly associated with the presence and concentration of *Salmonella* in market hog lymph nodes and tonsils?
3. What is the concentration of *Salmonella* in market hog lymph nodes that are positive?
4. Is there a difference in *Salmonella* enumeration methods 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®?
5. What serotypes of *Salmonella* are most frequently isolated from market hog lymph nodes and are these serotypes an epidemiological concern?

References

Acheson, D., & Hohmann, E. L. (2001). Nontyphoidal salmonellosis. *Clinical infectious diseases*, *32*(2), 263-269. https://doi.org/10.1086/318457

Bonilla, K. C. P. (2021). *Validation of commercial antimicrobial intervention technologies to control Salmonella and*

Shiga toxin-producing Escherichia coli (STEC) on pre-rigor, skin-on market hog carcasses and chilled pork wholesale cuts Kansas State University]. K-REx. https://krex.k-state.edu/bitstream/handle/2097/41364/KatiaCeciliaPozueloBonilla2021.pdf?sequence=1&isAllowed=y

CDC. (2019, 17 December 2019). *Foodborne infections*. CDC. https://www.cdc.gov/ncezid/what-we-do/our-topics/foodborne-disease.html#:~:text=According%20to%20CDC%20estimates%2C%20the,%2C%20Campylobacter%2C%20and%20Staphylococcus%20aureus.

CDC. (2022, September 9, 2022). *Serotypes and the importance of serotyping*. CDC. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html

CDC. (2023, 23 May 2023). *Salmonella*. Centers for Disease Control and Prevention. https://www.cdc.gov/salmonella/index.html

Chaves, B. D., Ruiz, H., Garcia, L. G., Echeverry, A., Thompson, L., Miller, M. F., & Brashears, M. M. (2017). High prevalence of *Salmonella* in lymph nodes and tonsils of swine presented for slaughter in Mexico. *Food protection trends*, *37*(1), 25.

Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella* enterica serovars differ. *Frontiers in microbiology*, *5*, 391-391. https://doi.org/10.3389/fmicb.2014.00391

Giannella, R. A. (1996). *Medical microbiology* (S. Baron, Ed. 4th ed.) https://www.ncbi.nlm.nih.gov/books/NBK8435/

Gragg, S. E., Loneragan, G. H., Brashears, M. M., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Wang, R., Schmidt, J. W., Brooks, J. C., Shackelford, S. D., Wheeler, T. L., Brown, T. R., Edrington, T. S., & Brichta-Harhay, D. M. (2013). Cross-sectional study examining *Salmonella* enterica carriage in subiliac lymph nodes of cull and feedlot cattle at harvest. *Foodborne pathogens and disease*, *10*(4), 368-374. https://doi.org/10.1089/fpd.2012.1275

Gut, A. M., Vasiljevic, T., Yeager, T., & Donkor, O. N. (2018). *Salmonella* infection - prevention and treatment by antibiotics and probiotic yeasts: a review. *Microbiology (Society for General Microbiology)*, *164*(11), 1327-1344. https://doi.org/10.1099/mic.0.000709

McQuiston, J. R., Waters, R. J., Dinsmore, B. A., Mikoleit, M. L., & Fields, P. I. (2011). Molecular determination of H antigens of *Salmonella* by use of a microsphere-based liquid array. *Journal of Clinical Microbiology*, *49*(2), 565-573. https://doi.org/10.1128/JCM.01323-10

Self, J. L., Luna-Gierke, R. E., Fothergill, A., Holt, K. G., & Vieira, A. R. (2017). Outbreaks attributed to pork in the United States, 1998-2015. *Epidemiology & Infection*, *145*(14), 2980-2990. https://doi.org/10.1017/s0950268817002114

USDA. (2023, 22 March 2023). *What is the most consumed meat in the world?* US Department of Agriculture. https://ask.usda.gov/s/article/What-is-the-most-consumed-meat-in-the-world

Wain, J., House, D., Zafar, A., Baker, S., Nair, S., Kidgell, C., Bhutta, Z., Dougan, G., & Hasan, R. (2005). Vi antigen expression in *Salmonella* enterica serovar Typhi clinical isolates from Pakistan. *Journal of Clinical Microbiology*, *43*(3), 1158-1165. https://doi.org/10.1128/JCM.43.3.1158-1165.2005

Zhang, Y., O'Connor, A. M., Wang, C., Dickson, J. S., Hurd, H. S., & Wang, B. (2019). Interventions targeting deep tissue lymph nodes may not effectively reduce the risk of salmonellosis from ground pork consumption: a quantitative microbial risk assessment. *Risk analysis*, *39*(10), 2237-2258. https://doi.org/10.1111/risa.13317

Chapter 2 - Literature Review

2.1 *Salmonella* background information

Foodborne outbreaks can be caused by several types of microorganisms, including bacteria, viruses, protozoa, and fungi. Bacteria cause the most cases of foodborne illness in humans (Possebon et al., 2021). Of these microorganisms, the Centers for Disease Control and Prevention (CDC) report that some of the most common foodborne outbreak causes are norovirus, *Salmonella*, and *Campylobacter*, to name a few (CDC, 2019). Foodborne disease can come from many sources; the most relevant being raw animal product, but other sources can be improperly cooked or handled foods, and contaminated fruits and vegetables (CDC, 2019).

The burden of foodborne disease in the United States is estimated to affect 48 million people each year, with 128,000 illnesses escalating to hospitalization, and causing 3,000 deaths (CDC, 2019). Statisticians estimate that the world population is expected to grow in the next decades, so food safety and security continue to be an important challenge to assess (Matthews et al., 2017). While educating consumers on proper food handling and cooking techniques may be beneficial, meat packing facilities have a duty to reduce pathogen load, which can be aided through routine plant screening and research.

Salmonellosis is one of the most common foodborne illnesses in the world (WHO, 2022). In the United States, *Salmonella* is estimated to infect 1.35 million people, with 26,500 hospitalizations and 420 deaths each year (CDC, 2023). *Salmonella* can be invasive, meaning that the infection can spread into the blood of the host (CDC, 2022d). Worldwide, gastroenteritis caused by non-typhoidal *Salmonella* is estimated to affect 93.8 million people each year, causing 155,000 deaths (Gong et al., 2022). The annual incidence rate is 15.2 cases per 100,000 people for salmonellosis (CDC, 2022a). This bacterium contributes to a considerable proportion of

foodborne illnesses each year, and understanding its characteristics is an important step in reducing cases. *Salmonella* is a rod-shaped, flagellated, Gram-negative, non-spore-forming, facultatively anaerobic bacterium, that is motile by means of peritrichous flagella (Giannella, 1996; Gut et al., 2018; Matthews et al., 2017). The *Salmonella* bacterium was named after Dr. Daniel Salmon and Theobald Smith; the serotype these researchers isolated is known today as *S. enterica* serotype Choleraesuis, and was found in hogs that were sick with swine fever (Knodler & Elfenbein, 2019). However, the early exploration of this pathogen began around the 1870s during which scientists were determined to understand typhoid fever in populated cities (Marineli et al., 2013).

Salmonella is notorious for being resilient and adaptive; the conditions required for growth and survival can be more extreme than other microorganisms. While cells are typically categorized as mesophilic, some strains can grow in temperatures higher or lower than the optimum 30-38˚C, and have been recorded to grow from a range of 2˚C to 54˚C (Matthews et al., 2017). The incubation temperatures used for the market hog LN prevalence, quantification, and serotyping studies was 37˚C - 42˚C depending on the growth medium (Vargas et al., 2023). *Salmonella* can also grow in a range of pH from 4.5 to 9.5, however optimum pH is closer to 7 (Matthews et al., 2017). In terms of seasonal variation in infections caused by *Salmonella*, more cases are reported during the summer months (i.e. June, July, and August) (CDC, 2022a). Furthermore, some research suggests an association with *Salmonella* presence and environmental factors such as increased rainfall and heat (Morgado et al., 2021).

Salmonella nomenclature

There are two species of *Salmonella*, *S. bongori* and *S. enterica*, with the latter being the species most associated with human salmonellosis (Matthews et al., 2017). *Salmonella enterica* can be further divided into six subspecies (also designated by a Roman numeral): *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI), respectively (Herrera-León et al., 2007; Matthews et al., 2017). More than half of all types of *Salmonella* fall into the *S. enterica* subsp. *enterica* group (Meštrović, 2023). Serotypes are named following the formula of a non-italicized serotype name with a capitalized first letter, and an italicized genus and species name (*e.g.*, *Salmonella enterica* Typhimurium). However not all serotypes have a name, so the antigenic formula from the White-Kauffmann-Le Minor scheme is used (Meštrović, 2023). *Salmonella* serotypes are often referred to by genus and serotype only (*e.g. Salmonella* Typhimurium) (Brenner et al., 2000).

Salmonella serotype

There are thousands of serotypes of *Salmonella*, which are characterized by surface structures of the cell (CDC, 2022d). There are three antigens that are associated with *Salmonella:* the O antigen that is located on the outer membrane on the surface of the cell (on the lipopolysaccharide layer, which is a fundamental part of Gram-negative bacterium), the H antigen that is determined by the flagellar protein, and the Vi antigen that is lies around the O antigen, which exists for few serotypes (CDC, 2022c; Giannella, 1996; McQuiston et al., 2011). The H antigen can be in phase 1 or phase 2, which are encoded by genes *fliC* and *fliB* (McQuiston et al., 2011). The genes responsible for flagellar phase expression are found on

different locations of the chromosome, however the genes cannot be expressed at the same time (Herrera-León et al., 2007). The third antigen is the Vi (capsular) antigen; *Salmonella* Typhi can contain the Vi antigen (Giannella, 1996), which is significant for pathogenesis as it increases infectivity and severity of disease (Wain et al., 2005). The somatic antigen is important for *Salmonella* grouping, while the flagellar antigen is important for serotype identity (Herrera-León et al., 2007). Strains belonging to serotypes Typhi, Paratyphi C, and Dublin are the only strains that have the Vi antigen (Matthews et al., 2017). Different serotypes and strains of *Salmonella* can induce a range of infections, however it is important to discuss secondary sequalae conditions such as arthritis, ankylosing spondylitis (fusion of spinal vertebrae), and Guillain-Barré syndrome (Esan et al., 2017; Matthews et al., 2017; MayoClinic, 2023). Serotypes can vary in terms of population around the world and the types of sources they contaminate; they also vary in severity of illness; different serotypes may have more prevalence in certain states in the U.S. (CDC, 2022d; Morgado et al., 2021).

Of the greater than 2,500 serotypes of *Salmonella*, only about 100 of them can cause disease in humans (Bonilla, 2021; CDC, 2023). Some examples of *Salmonella* serotypes that have been isolated from human cases are *S.* Enteritidis, Newport, Typhimurium, monophasic Typhimurium, Infantis, and Stanley (Ferrari et al., 2019; Matthews et al., 2017). The majority of identified serotypes come from the species *S. enterica* (Heithoff et al., 2008).The number of serotypes reported each year in clinical cases is relatively low, considering the sheer number of *Salmonella* serotypes. Furthermore, *Salmonella* can be host adapted: *S.* Typhi is human adapted, *S.* Gallinarium is adapted to poultry, *S.* Dublin is adapted to cattle, *S.* Choleraesuis is hog adapted, but *S.* Typhimurium and *S.* Enteritidis can infect any host (Gut et al., 2018).

Typhoidal *Salmonella* is a group of invasive serotypes that cause enteric fever, including *Salmonella* Typhi that causes typhoid fever, and *Salmonella* Paratyphi that causes paratyphoid fever (Gal-Mor et al., 2014). Enteric fever is more common in developing countries due to poor sanitation practices, leading to contamination by the fecal-oral route (Gal-Mor et al., 2014). The symptoms of enteric fever include fever, nausea and abdominal pain with constipation or diarrhea (WHO, 2023). The World Health Organization (WHO) estimates that 9 million people are affected by typhoid fever each year, with 110,000 deaths (WHO, 2023). Typhoidal salmonellae can be found in the bloodstream of those who are infected, and can persist and shed over a long period of time (Marineli et al., 2013; WHO, 2023). The well-known case of "Typhoid Mary" was the first documented asymptomatic carrier of typhoid fever in the U.S.; Mary contributed to the illnesses and deaths of nearly two dozen people by *Salmonella* Typhi while serving as a cook (Marineli et al., 2013). Antibiotic treatment is available for typhoid and paratyphoid fever, however success is variable due to the antibiotic resistance of some strains (Hughes et al., 2023).

Nontyphoidal *Salmonella* (NTS) serotypes are frequently associated with agricultural products and can cause human salmonellosis (Acheson & Hohmann, 2001). One source estimates there are more than 19,000 hospitalization each year caused by NTS in the U.S. (Morgado et al., 2021). Transmission of NTS strains can come from animal reservoirs, or from eating contaminated meat or produce, such as ground pork (Gong et al., 2022; Plumb et al., 2023). Salmonellosis caused by NTS is known to be a self-limiting (usually non-life-threatening compared to typhoidal salmonellae) illness that is associated with fever, abdominal cramps and pain, vomiting, and diarrhea (Knodler & Elfenbein, 2019). The Centers for Disease Control and Prevention (CDC) state that antibiotic treatment may be recommended for individuals with host

factors that put them at higher risk for infection, such as being under the age of 1, above the age of 65, or having a compromised immune system (CDC, 2022b). Recovery without treatment can take up to a week, however there are cases of NTS salmonellosis where illness can progress to more severe disease, such as bacteremia or focal invasive infection (Plumb et al., 2023).

Scientists believe that the widespread use of commercial antibiotics in both humans and livestock is contributing to the increase in antibiotic resistant strains of *Salmonella;* some strains have even become resistant to several types of antibiotics (Matthews et al., 2017; Possebon et al., 2021). Ampicillin and tetracyclines are some of the antibiotics that this pathogen can withstand. Some countries have banned the use of fluoroquinolones in animals over fear that this new antibiotic will be rendered useless against certain strains of *Salmonella* (Matthews et al., 2017). Another concern with antibiotic resistant strains of *Salmonella* is their potential to horizontally transfer genes encoded with resistance to other *Salmonella* strains, or potentially to other pathogenic bacteria (Possebon et al., 2021).

Salmonella pathogenicity and methods of infection

Host invasion is determined by host factors, such as age and immune status, as well as *Salmonella* serotype and virulence factors. Host response is also variable depending on the developmental level of the immune system, timing of an immune response, and composition of the gastrointestinal tract (Matthews et al., 2017). The ability of *Salmonella* to attach using fimbriae and invade host cells will be determined by virulence factors, and ultimately the chromosomes and plasmids (Giannella, 1996; Tan et al., 2016). There are several virulence factors possessed by *Salmonella* cells that determine pathogenesis: the capacity to invade and multiply in host cells, and composition of the lipopolysaccharide coat on the *Salmonella* cell; the

O-antigen on the lipopolysaccharide layer has influence on pathogenicity (Giannella, 1996; Gut et al., 2018). Interestingly, antimicrobial resistance genes are located on the same plasmid as genetic material for virulence factors (Matthews et al., 2017). One important virulence factor is the *Salmonella* pathogenicity island 1 (SPI-1)—the genes associated with pathogenicity island are responsible for factors such as host invasion, regulation of host inflammatory response, and biofilm production (Lou et al., 2019). The SPI-1 controls effector protein action in the type III protein secretion system; effector proteins are injected into host cells to control host immune response (Lou et al., 2019). Flagella are considered another virulence factor because as motility increases, so does invasiveness (Gut et al., 2018). Moreover, another virulence factor of *Salmonella* is the ability to produce toxins, which inhibit host responses such as protein synthesis (Giannella, 1996)

Salmonella may produce biofilms, which are a grouping of cells that survive under a self-produced extracellular polymeric substance that can attach to biotic or abiotic surfaces (Harrell et al., 2021; Nikolaev et al., 2022). The generation of biofilms can be de described in five steps following a negative external stimuli: reversible attachment, irreversible attachment, production of the extracellular polymeric substance, maturation, and dispersal (Muhammad et al., 2020). Biofilms serve as a survival mechanism and are created in response to negative environmental changes, such as nutrient availability and pH (Harrell et al., 2021). Bacteria within a biofilm can evade UV light, antibiotics, disinfectants, as well as host defenses (Harrell et al., 2021; Nikolaev et al., 2022). The ability of *S.* Typhi to survive in hosts for long periods of time (i.e. asymptomatic carriers) is related to the biofilm formation on gallstones in the gallbladder (Harrell et al., 2021). Unfortunately for the food industry, this means pathogens that generate biofilms can survive on surfaces following sanitation and be a cross-contamination source for the

surrounding environment (Nikolaev et al., 2022). Biofilms can form on many different surfaces such as stainless steel and glass, but most notably, biofilms can form on the surfaces of food itself (Harrell et al., 2021).

The infectious dose of *Salmonella* can range as low as a few cells according to an investigation of several food related outbreaks (Matthews et al., 2017), though infectious dose often varies by serotype. One source discussed the differences in infectious dose for typhoidal and non-typhoidal *Salmonella:* typical NTS strain infectious dose is around 3 logs, while the typhoidal strains infectious dose is closer to 5 logs (Canada, 2010). Furthermore, another study states 5 to 6 logs are needed to establish infection (Gut et al., 2018). Again, the number of cells required to cause infection will vary depending on host characteristics, but also serotype and virulence characteristics of the *Salmonella* cells.

Humans have defense mechanisms that are meant to prevent colonization of pathogens in the gastrointestinal system. However, *Salmonella* cells can evade host defenses such as acidity in the gut and sloughing of intestinal cells (Matthews et al., 2017). One of the most important virulence factors that *Salmonella* possesses is a type III protein secretion system, which is responsible for delivering effector proteins into the host cell that cause intestinal invasion (Lou et al., 2019). The type III protein secretion system is located on pathogenicity island 1 and is required for inflammation of the intestinal cells (Lou et al., 2019). The process of a *Salmonella* infection begins with the ingestion of cells, either by ingestion of contaminated food or water, or person-to-person contact (Giannella, 1996). *Salmonella* will colonize in the distal ileum and proximal colon (Gut et al., 2018). Using flagella, *Salmonella* will attach to M cells or on enterocytes (Gut et al., 2018). Following attachment, a type III secretion system will begin to inject effector proteins and toxins (Feldman, 2009). This is where the *Salmonella* pathogenicity

island 1 is utilized— cells are phagocytized by host macrophages or dendritic cells (Gut et al., 2018; Li, 2022). *Salmonella* cells invade the intestinal wall by causing ruffling of the intestinal membrane, where the intestinal cells will engulf the bacterium (Feldman, 2009; Giannella, 1996; Matthews et al., 2017). Then, cells begin to multiply in the epithelium and lymph tissue in the colon (Giannella, 1996). Replication of *Salmonella* cells occurs in the vacuole of the host cells, where is it safe from host defenses (Feldman, 2009; Matthews et al., 2017). During multiplication, *Salmonella* spreads to mesenteric lymph nodes and other parts of the body by dendritic cells (Giannella, 1996; Li, 2022). Lymph tissues contain membranous (M) cells on their surface, and *Salmonella* can destroy M cells to enter LNs (Li, 2022). Once the infection is established in the gut, the host inflammatory response is triggered (Feldman, 2009). *Salmonella* attachment to intestinal cells causes a release of pro-inflammatory cytokines—this is what will cause diarrhea and destruction of host mucosa cells (Feldman, 2009; Gut et al., 2018). Host inflammation allows the pathogen to outgrow the normal gut flora (Palmer & Slauch, 2017).

Public health and economic impact of *Salmonella*

The USDA Economic Research Service measures roughly $15.5 billion in economic strain for the top 15 pathogens that are tracked, with *Salmonella* being in the top five of those pathogens (Hoffmann, 2015). The economic burden associated with *Salmonella* in the United States is substantial: the USDA ERS estimates that the total impact from salmonellosis infections alone is $3.7 billion (USDA-ERS, 2015). This accounts for medical costs and financial losses from being ill, however most of the monetary burden lies with those who have died from a *Salmonella* infection. In general, there are several economic burdens that come from foodborne

illnesses such as wages lost from being ill or taking care of an ill person, time lost to recovery, and medical treatment costs (Hoffmann, 2015).

There has been more pressure on the pork industry about *Salmonella* reduction because big strides have been made in the poultry industry, especially in the European Union (Arguello et al., 2013). A total of 72 outbreaks of *Salmonella* can be attributed to pork products between the years of 1998 and 2015, which resulted in 2,215 illnesses, 276 hospitalizations, and no reported deaths (Self et al., 2017). In comparison, between the years 1990 and 2005, *Salmonella* in pork products were involved with 25% of the foodborne outbreaks and 36% of the human salmonellosis cases (Arguello et al., 2013).

Salmonella in swine production

It is common for livestock to harbor pathogens that can cause foodborne diseases such as *Salmonella*. Due to the methods for raising market hogs, commingling animals are likely to spread infections to other hogs (Miller et al., 2022). Disease occurs by ingestion of *Salmonella* contaminated food or water, or by direct contact (Knodler & Elfenbein, 2019). Between hogs, the most common transmission of pathogens occurs by the fecal-oral route (Bonardi, 2017). Hogs are at risk of contracting *S*. Typhisuis and *S*. Choleraesuis, which can be a fatal infection to hogs (Bonardi, 2017). Numerous studies report that livestock are recognized harbors of pathogens, such as *Salmonella,* that can lead to human illness (CDC, 2022b). Hogs that harbor *Salmonella* can be in a clinical or carrier state, meaning they can show symptoms of disease, or carry it without having clinical symptoms; hogs in a carrier state can spread infection to other hogs and potentially contaminate products at pork abattoirs (Lo Fo Wong et al., 2002). Asymptomatic carriage of *Salmonella* can occur following infection, where the pathogen can survive in tonsils,

lymphoid tissues in the gut, and in the gastrointestinal tract (Bonardi, 2017). Chaves et al. (2017) explored the idea of asymptomatic carriage of *Salmonella* in the mesenteric LNs, suggesting on farm exposure prior to harvest (Chaves et al., 2017).

Salmonella shedding from hogs has been known to increase in times of stress; stress factors include transportation from the finishing barns to the slaughterhouse, withdrawal from feed, and rough handling of hogs in lairage (Arguello et al., 2013; Bonardi, 2017; Chaves et al., 2017). The topic of lairage has been explored in several studies, where *Salmonella* shedding is believed to increase with longer lairage time and transportation stress (Bonardi, 2017; Hurd et al., 2001). Longer lairage time allows *Salmonella* more opportunity to invade mesenteric lymph nodes, especially after 12 hours (Bonardi, 2017). Confining hogs to enclosed spaces and commingling of hogs from different barns at lairage increases the risk of contaminating hogs with *Salmonella* before slaughter (Arguello et al., 2013). One study reports that there is enough time for *Salmonella* to colonize the gut during lairage, which can last up to 24 hours (or potentially longer) after unloading (Arguello et al., 2013). Moreover, Vieira-Pinto et al. (2012) reports that *Salmonella* can spread to tonsils in under half an hour after consumption; after invasion, it can spread to other lymphoid tissues rapidly (Vieira-Pinto et al., 2012). Other factors to consider for contamination of the hogs would be the environment that hogs are held in for lairage; improperly sanitized holding pens and dirty water troughs may contribute to *Salmonella* spread among hogs during lairage time (Arguello et al., 2013; Chaves et al., 2017).

Pork processing

During pork harvesting, there are several procedures used to reduce pathogen load as the carcasses move through the abattoir. Pork facilities use systems such as a Hazard Analysis

Critical Control Point (HACCP) plan and sanitary dressing processes to reduce contamination before further carcass breakdown (Vargas et al., 2023). The process of pork harvesting includes stunning, bleeding, scalding, dehairing, singeing, polishing and shaving (Arguello et al., 2013). Scalding and singeing are two heat treatments steps, and singeing (due to flames) is very effective at pathogen reduction, however improper temperature of the scalding tanks (less than 62˚C) can lead to contamination of many carcasses with *Salmonella* (Arguello et al., 2013). Reductions in pathogen loads have been reported following measures taken to reduce the microbial loads on pig skins (Bonardi, 2017). However, equipment, like carcass splitters, can become contaminated during processing from a carcass with *Salmonella*. Cross contamination can occur and ultimately results in the final contamination of the carcass during harvest (Bonardi, 2017).

Following the initial hot side of pork processing, the breakdown of carcasses presents another challenge for *Salmonella* proliferation in the abattoir. Tissues, such as tonsils, abdominal and mandibular lymph nodes, and contents of the gastrointestinal tract, can spread contamination during further processing and fabrication of the carcass (Arguello et al., 2013). Hands, utensils such as knives or saws, and sheaths are important sources of cross contamination (Arguello et al., 2013; Bonardi, 2017). One study references 55 to 90% of carcass contamination occurs during evisceration, highlighting the importance of proper dressing procedures to reduce pathogen load (Arguello et al., 2013). Antimicrobials, such as peracetic acid, can be used to reduce pathogen loads, however the USDA-FSIS conducted a survey and discovered that about half of the facilities that produce raw intact and non-intact pork cuts do not use antimicrobials. Following the establishment of *Salmonella* performance standards, it is expected that half of these

establishments will begin to use antimicrobials if they do not meet the requirements (USDA-FSIS, 2022).

Salmonella in pork lymph nodes

Numerous studies have been conducted by researchers interested in pathogens within pork lymph nodes, and the impact they have on consumer food safety. Researchers have identified lymph nodes as a source of contamination in pork products, but ground pork has the highest risk of contamination from lymph nodes (Zhang et al., 2019). Lymph nodes can be found in trim from the carcass, which becomes ground pork. According to Zhang et al. (2019), comminuted pork is at a higher risk for contamination than pork chops or other intact cuts because several carcasses can contribute to one sample of ground pork (Chaves et al., 2017; Zhang et al., 2019). Lymph nodes can be found throughout the carcass, however not all LN types will be incorporated into comminuted pork (Buettner & Bode, 2012; Vargas et al., 2023). Visceral LNs, such as the mesenteric LNs, are discarded during evisceration, but peripheral LNs located in adipose tissues can be incorporated into ground product (Miller et al., 2022; Vargas et al., 2023). Considering the number and location of LNs in the carcass, removal of all LNs is not a viable option due to line speed and effort required (Vargas et al., 2023).

The presence of pathogens such as *Salmonella* in the LNs is related to their filtering function; they are part of the lymphatic system that has a role in the regulation of the immune system. Lymph nodes function as filters that sort through lymph fluid where bacteria can be located. With this process in mind, researchers have been able to isolate pathogenic bacteria from LNs. According to Vieira-Pinto et al. (2005), the lymphatic system could be a risk for spreading pathogens in the carcass by route of lymph fluid (Vieira-Pinto et al., 2005). Vieira-Pinto et al.

(2012) explains how *Salmonella* can move through the lymph system: following ingestion, there is a rapid invasion of the tonsils. From here, cells can travel to the mandibular LNs, then cervical LNs, and then to the tracheal region and bloodstream (Horter et al., 2003; Vieira-Pinto et al., 2012). Mann et al. (2015) describes dendritic cells as the primary carrier for moving microbes from the gastrointestinal tract to lymph tissues. Dendritic cells (DCs) play a key role in filtering pathogens in the lymph fluid by picking up microbes, such as *Salmonella*, from intestinal or M cells. The dendritic cells can then carry microbes to LNs once they pass though mucosal sites in the gastrointestinal tract (Mann et al., 2015).

One lymphatic study indicated that the LNs located near the gastrointestinal tract interact with pathogens frequently (Buettner et al., 2011). Pathogens often enter the body through the oral route; this is related to the way that hogs interact with their environment. The lymphatic organs in the mouth and gastrointestinal tract use antigens to decide what to allow in the body or what warrants a reaction. Lymph nodes located near the gastrointestinal tract had the highest occurrence of *Salmonella* in comparison to the other LNs analyzed in a study conducted by Vieira-Pinto et al. (Vieira-Pinto et al., 2005). Due to their location in the head of a pig, tonsils are some of the first lymphatic tissues to interact with pathogens entering the body. *Salmonella* can colonize hog tonsils, and one study noted the ability of *S*. Typhimurium to colonize specifically in the tonsils (Horter et al., 2003).

Miller et al. (2022) conducted a preliminary evaluation of several types of pork LNs investigating prevalence in one pork processing plant (this study was the basis for the research study conducted in Chapter 3 of this thesis). This study found that *Salmonella* was present in all four types of LN samples collected in the study, with a reported prevalence of 34% in mesenteric LNs, inguinal with 18%, subiliac LN with 18%, and tracheobronchial with 16% prevalence of

Salmonella (Miller et al., 2022). A market hog LN study conducted in two abattoirs in Mexico analyzed four sample types that were collected, including tonsils, mandibular LNs, mesenteric LNs, and subiliac LNs. Prevalence ranged from 10.2% to 44.4% positive samples for *Salmonella*, with prevalence being the highest for mesenteric LNs in Merida, Mexico (Chaves et al., 2017). Moreover, market sow cervical and mandibular LNs have also been evaluated for *Salmonella* and reportedly had a prevalence of 0.9% and 16%, respectively, in one study. These studies again provide evidence that lymphoid tissues near the gastrointestinal system (including the mouth regarding tonsils and mandibular LNs) tend to have a higher prevalence of *Salmonella* (Harvey et al., 2020).

2.2 Overview of the lymph system

In 1879, Karl Joseph Eberth discovered *Salmonella* in abdominal LNs and in the spleen of patients suffering from typhoid fever (Marineli et al., 2013). This presentation of *Salmonella* in lymph tissues occurs not only in humans, but also in livestock (Chaves et al., 2017; Miller et al., 2022). The lymphatic system plays a critical role in protecting the health of the body through immune surveillance (Cueni & Detmar, 2008). There are several tissues and organs that are part of the lymphatic system including bone marrow, thymus, Peyer's patches, LNs, tonsils, and the spleen (Buettner & Bode, 2012; Cueni & Detmar, 2008). The lymph fluid is carried through the body during normal movements like walking and breathing, allowing for a constant filtering process (Cueni & Detmar, 2008). Lymph nodes are invaluable for reacting to pathogens like *Salmonella* by filtering lymph fluid to trap bacteria and viruses to remove them from the immune system. If pathogenic antigens are found while the lymph fluid is filtered, then an immune response will be produced (Buettner & Bode, 2012).

Lymphocytes (T and B cells) and dendritic cells are immune cells that aid LNs in the immune response and are transported through lymph fluid (Cueni & Detmar, 2008; Sainte-Marie, 2010). The two tandem afferent and efferent transport systems are responsible for moving T cells, B cells, and generated antibodies (Buettner & Bode, 2012). Using the afferent system, dendritic cells carry the antigen detected information to T cells. Activated T lymphocytes relay information to B cells, and once activated will determine which type of antibody to create in response to pathogen detection in the form of effector cells, which move via the efferent transport system (Buettner & Bode, 2012). Dendritic cells are constantly sampling for microbes; however, they are ineffective in their ability to kill bacteria. Therefore, movement of pathogens such as *Salmonella* to lymph nodes can occur asymptomatically (Mann et al., 2015).

2.3 Overview of PCR

Polymerase chain reaction (PCR) is a method used to create an exponential number of copies of a segment of DNA (Matthews et al., 2017). This technology can be used to amplify pathogen DNA to be able to detect the presence of a pathogen. Polymerase chain reaction has an essential role in food safety; it can be used as a fast and reliable method to both detect and quantify pathogens in food products with good specificity and sensitivity (Behlke, 2019).

Using several heat cycles and chemical processes, PCR can be described in three steps: the initial step involves denaturing the template DNA from its double helix structure to a single strand form. Once in the denatured form, segments of DNA that complement the template DNA (called primers) can attach to the segment of nucleotides of interest called annealing. Then, DNA polymerase can extend the attached primers known as elongation; these steps are repeated to

eventually create millions of copies of DNA (Matthews et al., 2017). This end point PCR technique gives a qualitative result for the target detection using the amplified solution.

Real Time PCR, or qPCR, is a technique used for quantification of a target. The qPCR machine is constantly measuring the level of fluorescence that is generated during DNA replication. Once a threshold has been reached for the level of fluorescence, the sample will be marked as positive, and the time to positive (measured in cycles) will aid in determining the enumeration of the target (Hygiena, 2018).

For *Salmonella*, the PCR systems are looking for the *invA* gene, which is considered the gold standard for *Salmonella* detection (González-Escalona et al., 2009; Yanestria et al., 2019). This gene has base pair sequences that are only found in *Salmonella* serotypes. One study investigating the presence of the *invA* gene in several pathogens (including *Salmonella*, *Shigella*, and *E.coli*) found that it was only present in the *Salmonella* genus (Pererat & Murray, 2008; Yanestria et al., 2019). Testing for this gene using PCR creates a more sensitive, fast, and reliable method for identifying *Salmonella* in samples when compared to traditional detection methods (Yanestria et al., 2019).

BAX quantification from PCR

A real time PCR method that can be used to detect and quantify *Salmonella* is the Hygiena™ BAX® Real Time *Salmonella* PCR Assay. This system is the PCR method used for *Salmonella* detection and quantification in the following chapters. The advantages of using this protocol include time, cost, and large sample capacity. There are several validated matrices that can be used across the meat industry for food safety testing, including pork LNs (Hygiena, 2021; Vargas et al., 2023). The traditional methods for serotyping are time consuming and require

many materials in the lab. *Salmonella* serotypes are categorized by their antigenic formula, which can be found on the White-Kauffmann-Le Minor scheme, which is updated by the World Health Organization (Meštrović, 2023). Molecular PCR has proven to be a powerful and resourceful tool for identifying specific genes in *Salmonella* for serotyping (Herrera-León et al., 2004).

The Hygiena™ BAX® PCR system uses PCR tablets that contain the required components for the reaction, which eliminates the need for traditional preparation of materials. Specific to the BAX® Real-time PCR system, there are probes that contain dyes called quencher and reporter dyes. During the elongation of the primer from DNA polymerase, the reporter dye is cleaved from the probe, which can then fluoresce as it is freed from the quencher dye. The PCR system searches for an increase in fluorescence, and once the threshold is reached will give a positive result for detection (Hygiena, 2018).

BAX®-System-SalQuant® is a new quantitative method that has been developed using the real-time PCR system and statistical analysis to quantify *Salmonella*. There has been more pressure placed on the meat industry to reduce *Salmonella* contamination, and a faster and more reliable quantification method is beneficial. Traditionally, the most probable number (MPN) method has been used to quantify pathogens such as *Salmonella* (Hygiena, 2020). Briefly, the MPN method can be described as a series of dilutions containing microbes that are evaluated for turbidity. Depending on the number of positive dilution tubes, a MPN value can be assigned (Sutton, 2010). While this method is reliable for samples with low levels of contamination and high background flora, MPNs require a lot of materials, time, and effort (Hygiena, 2020; Sutton, 2010). Using the BAX® System Q7 machine and BAX® System Real-time PCR assay, quantification is determined based on total number of cycles necessary for detection, known as

the cycle threshold (CT) value (Hygiena, 2020). As the CT value increases, the number of colony forming units (CFUs) decrease because it took more cycles to detect the targeted DNA. Once a CT value is determined, a calculation is performed based on the sample type to determine the quantification of the pathogen; the calculator was created from curve development and statistically analyzed through linear regression (Vargas et al., 2023).

In a study by Vargas et al. (2023), the BAX®-System-SalQuant® rapid enumeration technology for pork LNs was compared to MPN the more frequently used enumeration method of and 3M™ Enterobacteriaceae (EB) Petrifilm™ + Xylose Lysine Desoxycholate (XLD) replica plate method. Briefly, a 1 mL aliquot was plated on the EB Petrifilms (3M, Saint Paul, MN) in duplicate and incubated at 37˚C for 24 hours. Next, the incubated Petrifilms were placed agar side down on xylose lysine desoxycholate (XLD; Remel, Lenexa, KS), and pushed gently to transfer colonies to create the replica plates. The XLD plates were incubated for 18-24 hours at 37˚C, and characteristic *Salmonella* (black) colonies were counted (Vargas et al., 2023). The results from the evaluation discussed the limitations of all methodologies. Briefly, linear regression displayed that at six hours of incubation, BAX®-System-SalQuant® did a better job at estimating values less than 3 logs of *Salmonella*. Conversely, 3M™ EB Petrifilm™ + XLD replica plate method did a better job at estimating values that were greater than 3 logs of *Salmonella* (Vargas et al., 2023). In terms of evaluating LN *Salmonella* contamination, these data suggest that BAX®-System-SalQuant® can be an effective tool for enumerating LNs, which are a source of ground pork contamination.

2.4 Government standards for *Salmonella* (USDA)

The European Union has implemented governmental controls for *Salmonella* in pork products, and these regulations are heavily focused on on-farm production (Arguello et al., 2013). Several other countries have implemented similar *Salmonella* testing programs, and soon the USDA-FSIS will have new *Salmonella* performance standards for intact and non-intact pork products.

The USDA-FSIS estimates that pork accounts for 8-10% of *Salmonella* infections each year (USDA-FSIS, 2020). It is important for pork processing facilities to be aware of contamination sources for comminuted products since the USDA-FSIS will be evaluating them for *Salmonella* (USDA-FSIS, 2022). Once these new standards are finalized, they will replace the Raw Pork Sampling Program that is being used to monitor the presence of *Salmonella* in several types of pork products (USDA-FSIS, 2019). Proposed in 2015, the aim of the Raw Pork Sampling Program was to sample raw intact and non-intact pork cuts, and comminuted pork for pathogens across slaughter and retail establishments in two phases to establish a baseline for pathogen presence (USDA-FSIS, 2019).

The USDA-FSIS inspects several types of pork processing facilities including 1,110 slaughter facilities, 1,070 facilities that create pork cuts, and 1,334 facilities that make ground pork. Facilities that produce pork cuts and comminuted pork are the main targets of the standards; performance standards will be enforced for establishments that generate more than 50,000 pounds of pork cuts in a day, and for establishments generating more the 6,000 pounds of ground pork in a day. Moreover, there are 38 facilities that account for 91% of raw intact product and 138 facilities that account for nearly 96% of raw non-intact product (USDA-FSIS, 2020). Raw comminuted product has a higher risk of being contaminated with pathogens than whole

muscle cuts (Zhang et al., 2019). Contamination of ground pork can come from the combination of trim from many different carcasses, contamination from the grinding process, and incorporation of LNs (Zhang et al., 2019). In a recent update to USDA-FSIS guidelines to control *Salmonella* in the pork industry, USDA-FSIS cites a multiple-hurdle intervention approach for reducing *Salmonella* contamination that includes lymph node removal. Other hurdles mentioned include sanitation measures, slaughter process decontamination, antimicrobial interventions, and knife trimming and shaving (USDA-FSIS, 2023). Following industry testing, more than 30% of the tested raw non-intact product sampled was positive for *Salmonella*, in comparison to around 9% positive samples from raw intact samples (USDA-FSIS, 2020).

The new standards propose that in the span of one year, a plant producing comminuted pork is allowed to have a limited number of *Salmonella* positive samples out of the fifty-two total samples in a year. The range of allowable positive samples varies depending on the size of the plant. Those that do not meet this standard will have corrective actions and costs associated with attempting to meet this requirement following corrective actions (USDA-FSIS, 2022). The USDA-FSIS has a goal of decreasing salmonellosis cases by 25% and calculates that, to do so, only 13 out of 52 samples in a year can be *Salmonella* positive for comminuted pork. Similarly, only 6 out of 52 samples in a year can be positive for raw intact cuts. The USDA-FSIS estimates that around 56% of the raw non-intact facilities and 61% of raw intact facilities will be able to pass these performance standards once they have begun testing (USDA-FSIS, 2020). Adherence to performance standards will be evaluated on the past 52-week testing period for *Salmonella* detection (USDA-FSIS, 2022).

Testing for pathogen can be an expensive cost to endure, especially if facilities lack testing procedures already. Including sampling material and lost product, the USDA-FSIS

estimates that it will cost between $92,000 and $150,000 per year to evaluate adherence to performance standards (USDA-FSIS, 2022).

References

Acheson, D., & Hohmann, E. L. (2001). Nontyphoidal salmonellosis. *Clinical infectious diseases*, *32*(2), 263-269. https://doi.org/10.1086/318457

Arguello, H., Álvarez-Ordoñtez, A., Carvajal, A., Rubio, P., & Prieto, M. (2013). Role of slaughtering in *Salmonella* spreading and control in pork production. *Journal of food protection*, *76*(5), 899-911. https://doi.org/https://doi.org/10.4315/0362-028X.JFP-12-404

Behlke, M. A. (2019). *Polymerase chain reaction: theory and technology*. Caister Academic Press.

Bonardi, S. (2017). *Salmonella* in the pork production chain and its impact on human health in the European Union. *Epidemiology and infection*, *145*(8), 1513-1526. https://doi.org/10.1017/S095026881700036X

Bonilla, K. C. P. (2021). *Validation of commercial antimicrobial intervention technologies to control Salmonella and*

Shiga toxin-producing Escherichia coli (STEC) on pre-rigor, skin-on market hog carcasses and chilled pork wholesale cuts Kansas State University]. K-REx. https://krex.k-state.edu/bitstream/handle/2097/41364/KatiaCeciliaPozueloBonilla2021.pdf?sequence=1&isAllowed=y

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan, B. (2000). *Salmonella* nomenclature. *Journal of Clinical Microbiology*, *38*(7), 2465-2467. https://doi.org/10.1128/jcm.38.7.2465-2467.2000

Buettner, M., & Bode, U. (2012). Lymph node dissection – understanding the immunological function of lymph nodes. *Clinical and experimental immunology*, *169*(3), 205-212. https://doi.org/10.1111/j.1365-2249.2012.04602.x

Buettner, M., Pabst, R., & Bode, U. (2011). Lymph node stromal cells strongly influence immune response suppression. *European journal of immunology*, *41*(3), 624-633. https://doi.org/10.1002/eji.201040681

Canada, P. H. A. o. (2010). *Pathogen safety data sheets: infectious substances – Salmonella enterica spp.* Government of Canada. https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/salmonella-enterica.html#note4

CDC. (2019, 17 December 2019). *Foodborne infections*. CDC. https://www.cdc.gov/ncezid/what-we-do/our-topics/foodborne-disease.html#:~:text=According%20to%20CDC%20estimates%2C%20the,%2C%20Campylobacter%2C%20and%20Staphylococcus%20aureus.

CDC. (2022a, 31 August 2022). *Information for healthcare professionals and laboratories*. https://www.cdc.gov/salmonella/general/technical.html#:~:text=Salmonella%20infection%20is%20more%20common,get%20a%20Salmonella%20infection3.

CDC. (2022b, September 9, 2022). *Questions & answers*. CDC. https://www.cdc.gov/salmonella/general/index.html#:~:text=How%20common%20is%20Salmonella%20infection,the%20United%20States%20every%20year.

CDC. (2022c, September 9, 2022). *Serotypes and the importance of serotyping*. CDC. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html

CDC. (2022d, 08 July 2022). *Snapshots of Salmonella serotypes*. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotype-snapshots.html

CDC. (2023, 23 May 2023). *Salmonella*. Centers for Disease Control and Prevention. https://www.cdc.gov/salmonella/index.html

Chaves, B. D., Ruiz, H., Garcia, L. G., Echeverry, A., Thompson, L., Miller, M. F., & Brashears, M. M. (2017). High prevalence of *Salmonella* in lymph nodes and tonsils of swine presented for slaughter in Mexico. *Food protection trends, 37*(1), 25.

Cueni, L. N., & Detmar, M. (2008). The lymphatic system in health and disease. *Lymphatic research and biology, 6*(3-4), 109-122. https://doi.org/10.1089/lrb.2008.1008

Esan, O. B., Pearce, M., van Hecke, O., Roberts, N., Collins, D. R. J., Violato, M., McCarthy, N., Perera, R., & Fanshawe, T. R. (2017). Factors associated with sequelae of *Campylobacter* and non-typhoidal *Salmonella* infections: a systematic review. *EBioMedicine, 15*(C), 100-111. https://doi.org/10.1016/j.ebiom.2016.12.006

Feldman, M. (2009, 30 March 2009). *Salmonella pathogensis* [Youtube video]. Youtube. https://www.youtube.com/watch?v=j5GvvQJVD_Y

Ferrari, R. G., Rosario, D. K. A., Cunha-Neto, A., Mano, S. B., Figueiredo, E. E. S., & Conte-Junior, C. A. (2019). Worldwide epidemiology of *Salmonella* serovars in animal-based

foods: a meta-analysis. *Applied and Environmental Microbiology*, *85*(14). https://doi.org/10.1128/AEM.00591-19

Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella* enterica serovars differ. *Frontiers in microbiology*, *5*, 391-391. https://doi.org/10.3389/fmicb.2014.00391

Giannella, R. A. (1996). *Medical microbiology* (S. Baron, Ed. 4th ed.) https://www.ncbi.nlm.nih.gov/books/NBK8435/

Gong, B., Li, H., Feng, Y., Zeng, S., Zhuo, Z., Luo, J., Chen, X., & Li, X. (2022). Prevalence, serotype distribution and antimicrobial resistance of non-typhoidal *Salmonella* in hospitalized patients in Conghua District of Guangzhou, China. *Frontiers in cellular and infection microbiology*, *12*, 805384-805384. https://doi.org/10.3389/fcimb.2022.805384

González-Escalona, N., Hammack, T. S., Russell, M., Jacobson, A. P., De Jesús, A. J., Brown, E. W., & Lampel, K. A. (2009). Detection of live *Salmonella* spp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting invA mRNA. *Applied and Environmental Microbiology*, *75*(11), 3714-3720. https://doi.org/10.1128/AEM.02686-08

Gut, A. M., Vasiljevic, T., Yeager, T., & Donkor, O. N. (2018). *Salmonella* infection - prevention and treatment by antibiotics and probiotic yeasts: a review. *Microbiology (Society for General Microbiology)*, *164*(11), 1327-1344. https://doi.org/10.1099/mic.0.000709

Harrell, J. E., Hahn, M. M., D'Souza, S. J., Vasicek, E. M., Sandala, J. L., Gunn, J. S., & McLachlan, J. B. (2021). *Salmonella* biofilm formation, chronic infection, and immunity within the intestine and hepatobiliary tract. *Frontiers in cellular and infection microbiology*, *10*, 624622-624622. https://doi.org/10.3389/fcimb.2020.624622

Harvey, R. B., Norman, K. N., Anderson, R. C., & Nisbet, D. J. (2020). A preliminary study on the presence of *Salmonella* in lymph nodes of sows at processing plants in the United States. *Microorganisms (Basel)*, *8*(10), 1602. https://doi.org/10.3390/microorganisms8101602

Heithoff, D. M., Shimp, W. R., Lau, P. W., Badie, G., Enioutina, E. Y., Daynes, R. A., Byrne, B. A., House, J. K., & Mahan, M. J. (2008). Human *Salmonella* clinical isolates distinct from those of animal origin. *Applied and Environmental Microbiology*, *74*(6), 1757-1766. https://doi.org/10.1128/AEM.02740-07

Herrera-León, S., McQuiston, J. R., Usera, M. A., Fields, P. I., Garaizar, J., & Echeita, M. A. (2004). Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *Journal of Clinical Microbiology*, *42*(6), 2581-2586. https://doi.org/10.1128/jcm.42.6.2581-2586.2004

Herrera-León, S., Ramiro, R., Arroyo, M., Díez, R., Usera, M. A., & Echeita, M. A. (2007). Blind comparison of traditional serotyping with three multiplex PCRs for the identification of *Salmonella* serotypes. *Research in Microbiology*, *158*(2), 122-127. https://doi.org/10.1016/j.resmic.2006.09.009

Hoffmann, S. (2015, 08 September 2015). *Quantifying the impacts of foodborne illnesses*. https://www.ers.usda.gov/amber-waves/2015/september/quantifying-the-impacts-of-foodborne-illnesses/

Horter, D. C., Yoon, K.-J., & Zimmerman, J. J. (2003). A review of porcine tonsils in immunity and disease. *Animal health research reviews*, *4*(2), 143-155. https://doi.org/10.1079/AHRR200358

Hughes, M., Appiah, G., & Watkins, L. F. (2023, 1 May 2023). *Typhoid & paratyphoid fever*. Centers for Disease Control and Prevention. https://wwwnc.cdc.gov/travel/yellowbook/2024/infections-diseases/typhoid-and-paratyphoid-fever

Hurd, H. S., McKean, J. D., Wesley, I. V., & Karriker, L. A. (2001). The Effect of lairage on *Salmonella* isolation from market swine. *Journal of food protection*, *64*(7), 939-944. https://doi.org/10.4315/0362-028X-64.7.939

Hygiena. (2018, 1 November 2018). *How real-time and end-point PCR works in the BAX® system* Youtube, Hygiena. https://www.youtube.com/watch?v=JeL77b1UyfU

Hygiena. (2020). *The BAX® System SalQuant introduces new era of accurately quantifying Salmonella levels*. Hygiena. https://www.hygiena.com/wp-content/uploads/2020/06/WPFB-Salquant-New-Era.pdf

Hygiena. (2021). *BAX® system Q7 user guide* [PDF]. Qualicon Diagnostics LLC. https://www.hygiena.com/wp-content/uploads/2021/05/BAX-System-Q7-Users-Guide.pdf

Knodler, L. A., & Elfenbein, J. R. (2019). *Salmonella* enterica. *Trends in microbiology (Regular ed.)*, *27*(11), 964-965. https://doi.org/10.1016/j.tim.2019.05.002

Li, Q. (2022). Mechanisms for the invasion and dissemination of *Salmonella*. *The Canadian journal of infectious diseases & medical microbiology*, *2022*, 1-12. https://doi.org/10.1155/2022/2655801

Lo Fo Wong, D. M. A., Hald, T., van der Wolf, P. J., & Swanenburg, M. (2002). Epidemiology and control measures for *Salmonella* in pigs and pork. *Livestock Production Science*, *76*(3), 215-222. https://doi.org/https://doi.org/10.1016/S0301-6226(02)00121-5

Lou, L., Zhang, P., Piao, R., & Wang, Y. (2019). *Salmonella* pathogenicity island 1 (SPI-1) and its complex regulatory network. *Frontiers in cellular and infection microbiology*, *9*, 270-270. https://doi.org/10.3389/fcimb.2019.00270

Mann, E., Dzieciol, M., Pinior, B., Neubauer, V., Metzler-Zebeli, B. U., Wagner, M., & Schmitz-Esser, S. (2015). High diversity of viable bacteria isolated from lymph nodes of slaughter pigs and its possible impacts for food safety. *Journal of applied microbiology*, *119*(5), 1420-1432. https://doi.org/10.1111/jam.12933

Marineli, F., Tsoucalas, G., Karamanou, M., & Androutsos, G. (2013). Mary Mallon (1869-1938) and the history of typhoid fever. *Annals of gastroenterology*, *26*(2), 132-134.

Matthews, K. R., Kniel, K. E., & Montville, T. J. (2017). *Food microbiology- an introduction (4th Edition)* (4th ed.). American Society for Microbiology (ASM).

MayoClinic. (2023, 21 February 2023). *Ankylosing spondylitis*. Mayo Foundation for Medical Education and Research. https://www.mayoclinic.org/diseases-conditions/ankylosing-spondylitis/symptoms-causes/syc-20354808#:~:text=Ankylosing%20spondylitis%2C%20also%20known%20as,be%20difficult%20to%20breathe%20deeply.

McQuiston, J. R., Waters, R. J., Dinsmore, B. A., Mikoleit, M. L., & Fields, P. I. (2011). Molecular determination of H antigens of *Salmonella* by use of a microsphere-based liquid array. *Journal of Clinical Microbiology*, *49*(2), 565-573. https://doi.org/10.1128/JCM.01323-10

Meštrović, T. (2023, 06 March 2023). *Salmonella nomenclature*. https://www.news-medical.net/health/Salmonella-Nomenclature.aspx

Miller, M., Maher, J. M., Wiseman, B., & Gragg, S. E. (2022). *Salmonella* is present in multiple lymph nodes of market hog carcasses at slaughter. *Food protection trends*, *42*(2), 100-106. https://doi.org/https://doi.org/10.4315/FPT-21-027

Morgado, M. E., Jiang, C., Zambrana, J., Upperman, C. R., Mitchell, C., Boyle, M., Sapkota, A. R., & Sapkota, A. (2021). Climate change, extreme events, and increased risk of salmonellosis: foodborne diseases active surveillance network (FoodNet), 2004-2014. *Environmental health*, *20*(1), 105-105. https://doi.org/10.1186/s12940-021-00787-y

Muhammad, M. H., Idris, A. L., Fan, X., Guo, Y., Yu, Y., Jin, X., Qiu, J., Guan, X., & Huang, T. (2020). Beyond risk: bacterial biofilms and their regulating approaches. *Frontiers in microbiology*, *11*, 928-928. https://doi.org/10.3389/fmicb.2020.00928

Nikolaev, Y., Yushina, Y., Mardanov, A., Gruzdev, E., Tikhonova, E., El-Registan, G., Beletskiy, A., Semenova, A., Zaiko, E., Bataeva, D., & Polishchuk, E. (2022). Microbial biofilms at meat-processing plant as possible places of bacteria survival. *Microorganisms (Basel)*, *10*(8), 1583. https://doi.org/10.3390/microorganisms10081583

Palmer, A. D., & Slauch, J. M. (2017). Mechanisms of *Salmonella* pathogenesis in animal models. *Human and Ecological Risk Assessment: An International Journal*, *23*(8), 1877-1892. https://doi.org/10.1080/10807039.2017.1353903

Pererat, K., & Murray, A. (2008). Development of a PCR assay for the identification of *Salmonella* enterica serovar Brandenburg. *Journal of medical microbiology*, *57*(10), 1223-1227. https://doi.org/10.1009/jmm.0.2008/002337-0

Plumb, I., Fields, P., & Bruce, B. (2023, 1 May 2023). *Salmonellosis, nontyphoidal*. Centers for Disease Control and Prevention. https://wwwnc.cdc.gov/travel/yellowbook/2024/infections-diseases/salmonellosis-nontyphoidal#:~:text=Nontyphoidal%20Salmonella%20infection%20usually%20presents,treatment%20after%201%E2%80%937%20days.

Possebon, F. S., Alvarez, M. V. N., Ullmann, L. S., & Araújo Jr, J. P. (2021). Antimicrobial resistance genes and class 1 integrons in MDR *Salmonella* strains isolated from swine lymph nodes. *Food control*, *128*, 108190. https://doi.org/10.1016/j.foodcont.2021.108190

Sainte-Marie, G. (2010). The lymph node revisited: development, morphology, functioning, and role in triggering primary immune responses. *Anatomical Record*, *293*(2), 320-337. https://doi.org/10.1002/ar.21051

Self, J. L., Luna-Gierke, R. E., Fothergill, A., Holt, K. G., & Vieira, A. R. (2017). Outbreaks attributed to pork in the United States, 1998-2015. *Epidemiology & Infection*, *145*(14), 2980-2990. https://doi.org/10.1017/s0950268817002114

Sutton, S. (2010). The most probable number method and its uses in enumeration, qualification, and validation. *Journal of validation technology*, *16*(3), 35.

Tan, M. S. F., White, A. P., Rahman, S., & Dykes, G. A. (2016). Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. *PloS one*, *11*(6), e0158311-e0158311. https://doi.org/10.1371/journal.pone.0158311

USDA-ERS. (2015, 05 January 2015). *Foodborne illnesses caused by Salmonella cost the U.S. an estimated $3.7 billion annually*. https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail/?chartId=77908#:~:text=Foodborne%20Salmonella%20infections%20impose%20an,to%20pay%20to%20prevent%20deaths.

USDA-FSIS. (2019). *Raw pork products exploratory sampling program*. https://www.fsis.usda.gov/science-data/sampling-program/raw-pork-products-exploratory-sampling-program

USDA-FSIS. (2020). *Public health effects of performance standards for comminuted pork and pork cuts*. Retrieved from https://www.fsis.usda.gov/sites/default/files/media_file/2022-02/Pork_Salmonella_Performance_Standards_Risk_Assessment_April_8_2020_Feb_8_2022.pdf

USDA-FSIS. (2022). *Preliminary cost-benefit analysis: proposed performance standards for Salmonella in raw comminuted pork and intact or nonIntact pork cuts*. Retrieved from https://www.fsis.usda.gov/sites/default/files/media_file/2022-02/Pork-Salmonella-Performance-Standards-Cost-Benefit-Analysis.pdf

USDA-FSIS. (2023, 01 June 2023). *FSIS guideline to control Salmonella in swine slaughter and pork processing establishments* US Department of Agriculture Food Safety and Inspection Service. https://www.fsis.usda.gov/sites/default/files/media_file/documents/FSIS-GD-2023-0003.pdf

Vargas, D. A., Betancourt-Barszcz, G. K., Blandon, S. E., Applegate, S. F., Brashears, M. M., Miller, M. F., Gragg, S. E., & Sanchez-Plata, M. X. (2023). Rapid quantitative method development for beef and pork lymph nodes using BAX ® system real time *Salmonella* Assay. *Foods*, *12*(4).

Vieira-Pinto, M., Temudo, P., & Martins, C. (2005). Occurrence of *Salmonella* in the ileum, ileocolic lymph nodes, tonsils, mandibular lymph nodes and carcasses of pigs slaughtered for consumption. *Journal of veterinary medicine. Series B*, *52*(10), 476-481. https://doi.org/10.1111/j.1439-0450.2005.00892.x

Vieira-Pinto, M., Tenreiro, R., Aranha, J., & Martins, C. (2012). Relationship between tonsils and mandibular lymph nodes concerning *Salmonella* sp. infection. *Food research international*, *45*(2), 863-866. https://doi.org/10.1016/j.foodres.2010.09.023

Wain, J., House, D., Zafar, A., Baker, S., Nair, S., Kidgell, C., Bhutta, Z., Dougan, G., & Hasan, R. (2005). Vi antigen expression in *Salmonella* enterica serovar Typhi clinical isolates from Pakistan. *Journal of Clinical Microbiology*, *43*(3), 1158-1165. https://doi.org/10.1128/JCM.43.3.1158-1165.2005

WHO. (2022, May 19, 2022). *Food safety*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/food-safety#:~:text=Salmonella%2C%20Campylobacter%20and%20enterohaemorrhagic%20Escherichia,with%20severe%20and%20fatal%20outcomes.

WHO. (2023, 30 March 2023). *Typhoid*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/typhoid#:~:text=Salmonella%20Typhi%20lives%20only%20in,patients%20may%20have%20a%20rash.

Yanestria, S. M., Rahmaniar, R. P., Wibisono, F. J., & Effendi, M. H. (2019). Detection of invA gene of *Salmonella* from milkfish (Chanos chanos) at Sidoarjo wet fish market, Indonesia, using polymerase chain reaction technique. *Veterinary World*, *12*(1), 170-175. https://doi.org/10.14202/vetworld.2019.170-175

Zhang, Y., O'Connor, A. M., Wang, C., Dickson, J. S., Hurd, H. S., & Wang, B. (2019). Interventions targeting deep tissue lymph nodes may not effectively reduce the risk of salmonellosis from ground pork consumption: a quantitative microbial risk assessment. *Risk analysis*, *39*(10), 2237-2258. https://doi.org/10.1111/risa.13317

Chapter 3 - Comparison of *Salmonella* Enumeration Methods and an Investigation of *Salmonella* Prevalence and Quantification in Market Hog Lymph Nodes and Tonsils in Several Regions and Seasons of the United States

*To be submitted to the Journal of Food Protection

Abstract

Market hog lymph nodes (LNs) can contaminate carcasses with *Salmonella,* as well as ground and comminuted pork products. Pork LNs pose a risk to food safety; therefore, it is advantageous to perform a qualitative and quantitative analysis of LNs from several regions and seasons in the U.S. to establish a baseline of prevalence, concentration, and distribution of *Salmonella* serotypes. Six types of LNs (axillary, mesenteric, subiliac, tracheobronchial, superficial inguinal, and pre-scapular) and tonsils were sampled from market hog carcasses from five pork processing facilities, located in three different regions (east, central, and west) over three seasons (winter, spring, and fall) for a total of 4,132 samples. Four facilities were visited once per season and one facility was visited twice per season. The samples were evaluated for *Salmonella* presence and concentration using BAX® System Real-Time *Salmonella* Assay and BAX®-System-SalQuant® methods, respectively. An enumeration method comparison was also conducted between 3M™EB-Petrifilm™ + XLD-replica plates, and BAX®-System-SalQuant®. *Salmonella* prevalence among sample types was 36% for tonsils, 35% for mesenteric LN, and less than 10% for the other LN types. Of the 601 carcasses tested, 62% were positive for *Salmonella,* with the highest prevalence occurring during the spring in the eastern region

(90.9%), and the lowest prevalence occurring during the spring in the central region (26.0%), in general. Prevalence varied according to region and season for tonsils (P=0.006), with a greater prevalence observed in the eastern region during spring. In general, mesenteric LN *Salmonella* prevalence was high (greater than 20%) regardless of season or region. In general, *Salmonella* prevalence in tracheobronchial, subiliac, axillary, and superficial inguinal LNs was greatest during the spring or fall and in the eastern region; however, statistical significance according to region, season, and the season by region interaction did vary for each sample type. The median values for *Salmonella* concentration for mesenteric, axillary, superficial inguinal, subiliac, tracheobronchial, and pre-scapular LNs fell below the limit of quantification for BAX®-System-SalQuant®. The median concentration for *Salmonella* in tonsils was of 2.18 $\log_{10}$ CFU/Sample. When comparing the 3M™ EB-Petrifilm™ + XLD-replica plates and BAX®-System-SalQuant® methods for quantification, statistical analysis determined that both methodologies can be used for market hog lymph node enumeration. The kappa coefficient between the two methods was 0.384, suggesting fair agreement between quantification using 3M™ EB-Petrifilm™ + XLD-replica plates and BAX®-System-SalQuant® methods. A t-test was performed on the 83 samples that were quantified by both methodologies, and it was observed that there was no significant difference in the mean log (CFU/sample) for the two methods (p=0.289). Efficacy between the methods may vary depending on sample type. This longitudinal study maps LN *Salmonella* prevalence and concentration within market hog carcasses throughout the U.S. and the data can be used by the pork industry for risk assessments and risk-based decision making.

3.1 Introduction

As of 2021, pork is the second most widely consumed type of meat in the world (Shahbandeh, 2022a), and third-most consumed type of meat in the U.S. (Shahbandeh, 2022b). Researchers have an epidemiological interest in exploring pathogen contamination in pork processing facilities to reduce foodborne illnesses linked to pork products. It was estimated that 110,510,000 metric tons of pork would be produced worldwide in 2022 (Shahbandeh, 2022c) and projected that 122,110 kilotons of pork would be consumed worldwide in 2023 (Shahbandeh, 2022d). However, the United States Department of Agriculture (USDA) estimates that pork products are responsible for more than 500,000 foodborne illnesses each year in the United States (Self et al., 2017). Between 1998 and 2015, there were 288 foodborne outbreaks linked to contaminated pork products (Self et al., 2017). While this data accounts for only a fraction of total foodborne illnesses in the U.S., more research needs to be conducted to reduce the number of foodborne illnesses from pork products.

One of the most common pathogens that causes foodborne illnesses in the world is *Salmonella* (Chaves et al., 2017; WHO, 2022); it is a rod shaped, Gram-negative, facultatively anaerobic bacterium that has two main species: *S. enterica* and *S. bongori* (Matthews et al., 2017). There are more than 2,500 *Salmonella* serotypes that have been identified, however not all stereotypes are pathogenic to humans; the species most associated with human and animal illness are *S. enterica* (Matthews et al., 2017). This Enterobacteriaceae bacterium causes salmonellosis, which can vary in severity depending on host and agent factors (Matthews et al., 2017). Salmonellosis is a medical and economic burden, and the Centers for Disease Control (CDC) estimates that each year *Salmonella* causes 1.35 million illnesses, 26,500 hospitalizations, and more than 400 deaths in the U.S. (2022b).

Between the years 1998 and 2015, there were 72 outbreaks of *Salmonella* attributed to pork products, resulting in 2,215 illness and 276 hospitalizations (Self et al., 2017). According to the CDC, the most recent pork-related *Salmonella* outbreak investigation occurred in 2015 (CDC, 2022a), spanning amongst 5 states with 192 illnesses (CDC, 2015). Research suggests that ground product is at risk of containing contaminated LNs (Arthur et al., 2008; Miller et al., 2022; Vargas et al., 2023). Lymph nodes are part of the immune system, and the role is to filter and subdue bacteria and viruses found in the body (Arthur et al., 2008; Vargas et al., 2023). During host invasion by *Salmonella*, cells can multiply and spread to LNs, most notably the mesenteric LN due to its location in the viscera (i.e. close proximity to the gut after cells are consumed) (Giannella, 1996). Exposure to *Salmonella* can occur during the harvest and breakdown of the carcass, either by machine or knife once the LN is sliced open. Due to normal methods of pork processing, trim (which consists of fat and muscle tissue) can contain LNs destined for use in comminuted pork (Chaves et al., 2017). For LNs that harbor *Salmonella* and are still encased in adipose tissue, they are protected from chemical and thermal interventions at the abattoir (Gragg et al., 2013; Vargas et al., 2023); thus, rendering normal harvest interventions an inadequate response to this type of contamination. Several studies support the idea of physical removal of the LNs from the carcass to address LN contamination (Bueno López et al., 2022; Miller et al., 2022).

Previous studies investigating *Salmonella* contamination in LNs report a range of prevalence. Miller et al. (2022) reported 34% mesenteric, 18.4% inguinal, 18.3% subiliac, and 16.3% tracheobronchial prevalence (Miller et al., 2022). Chaves et al. 2017 reported a 10% to 44% *Salmonella* prevalence among three pork LN types and tonsils (Chaves et al., 2017). While mesenteric LNs are discarded during processing, other LN types remain in the carcass and

untouched by harvest interventions, allowing a possible contamination source for ground product (Vargas et al., 2023).

Rapid pathogen enumeration methods are quickly becoming industry standard; knowing the concentration of pathogens entering processing facilities gives insight into risk of contamination (Mann et al., 2014; Vargas et al., 2023). Polymerase chain reaction (PCR) has become a more frequent method of pathogen quantification due to time and cost, when comparing to other more traditional methods of quantification, such as most probable number (MPN) or direct plating (Lei et al., 2021; Vargas et al., 2023).

The objectives of this study were to evaluate which LN type presented the highest risk for containing *Salmonella* and at what level of contamination, while also investigating the seasonal and regional variability of *Salmonella* in market hog LNs. Another objective of this study was to compare a direct plating method for enumeration (3M™ EB Petrifilm™ + XLD replica plate) with a rapid PCR method (BAX®-System-SalQuant®). Several authors have published their work related to prevalence among different pork LN types, however there is limited knowledge about regional variation. An even greater knowledge gap exists about seasonal variation in *Salmonella* distribution in market hog lymph nodes. With USDA pork *Salmonella* performance standards forthcoming, this study provides insight into the role of market hog LN *Salmonella* contamination.

3.2 Materials and methods

3.2.1 Sample collection

The study was conducted in pork processing facilities located in three regions of the United States, including west, central, and east. A total of five facilities were included in this

study. Four facilities were sampled once every season and one facility was sampled twice per season, for three seasons (winter, spring, and fall), for a total of 18 individual sampling events. The winter samplings occurred during February and March 2022; the spring samplings occurred in May and June of 2022; the fall samplings occurred during September and October of 2022. Seven sample types were collected from every market hog carcass, including mesenteric LN, subiliac LN, superficial inguinal LN, prescapular LN, axillary LN, tracheobronchial LN, and tonsils. From each plant location during each season, these seven sample types were taken from 30-35 carcasses. All carcasses were USDA Food Safety and Inspection Service (USDA-FSIS) inspected and passed. The LNs and tonsils were collected post-inspection but prior to chilling; samples were placed into individual sterile Whirl-Pak® bags (Nasco, Atlanta, GA). After leaving the harvest floor, samples were placed into insulated containers with cooler packs, and transferred to a 4˚C refrigerator for next day processing at either the K-State Food Safety and Defense Laboratory at Kansas State University or the International Center for Food Industry Excellence (ICFIE) Food Microbiology Laboratory at Texas Tech University.

3.2.2 Sample processing

Samples were processed and analyzed for *Salmonella* as described in the method development for BAX® system real time PCR (Vargas et al., 2023). Briefly, the LNs and tonsils were trimmed from any surrounding fat and fascia using scissors sanitized before each sample, weighed, and categorized (small: <3 grams; medium: >3 grams and <25 grams). Then, samples were submerged in boiling water (100˚C) for 3-5 seconds for surface sterilization, placed into an individual filtered sterile Whirl-Pak® bag, and pulverized with a rubber mallet. BAX® system MP media (Hygiena, Camarillo, CA) was added aseptically according to weight (small: 20 mL,

medium: 80 mL), and subjected to a stomacher (Biomerieux, Marcy-I'Etoile, France) at 620 strokes/min for 1 minute for homogenization. The LN homogenate will be referred to as LNH hereafter**.**

3.2.3 *Salmonella* enumeration

Two methods were used to enumerate *Salmonella* from LNS and tonsils: a direct plating method using Enterobacteriaceae (EB) Petrifilm (3M, Saint Paul, MN) + XLD replica plating (following methods described in Vargas et al. (2023)) and BAX®-System-SalQuant® (Hygiena, Camarillo, CA).

For 3M™ EB Petrifilm™ + XLD replica plate, a 1 mL aliquot was removed from each LNH to be plated on EB Petrifilm and incubated for 22-26 hours at 37˚C. Characteristic *Salmonella* growth on EB Petrifilm (reddish/purple colonies with acid and gas production) were counted, and then replica plated onto xylose lysine desoxycholate (XLD; Remel, Lenexa, KS) agar plates. The replica plate was prepared by placing the inoculated film from the EB Petrifilm onto an XLD plate and gently pushing to transfer colonies. XLD plates were incubated for 18-24 hours at 37˚C, the plate counts were recorded by counting characteristic black colonies as *Salmonella*.

For BAX®-System-SalQuant® samples were subjected to the BAX® System Real-time PCR Assay (Hygiena, Camarillo, CA) for *Salmonella* quantification following a 6 hour incubation at 42˚C (Vargas et al., 2023). Sample bags were hand-massaged for 15 seconds before a 5 µl aliquot was removed. The aliquot was processed following manufacturer guidelines for BAX®-System-SalQuant® for *Salmonella* enumeration and concentration was calculated as described by Vargas et al. (2023).

3.2.4 *Salmonella* detection

Following the enumeration procedures, sample bags were returned to the incubator for another 18 hours at 42˚C. For samples that were not positive during BAX®-System-SalQuant® at 6 hours, twenty-four-hour enrichments were hand massaged for 15 seconds before a 5 µl aliquot was removed and used for BAX® System RT-PCR Assay for *Salmonella* as a detection step.

3.2.5 *Salmonella* confirmation and isolation

Samples that were positive for *Salmonella* at 24-hours were subjected to culture isolation. From the LNH, a sterile loop was used to streak for isolation onto XLD agar plates, and incubated for 18-24 hours at 37˚C. Agar plates that contained colonies that were characteristic of *Salmonella* (black, round colonies) were then streaked for isolation on XLD plates, with up to three colonies selected per plate, and incubated for 18-24 hours at 37˚C (Aryal, 2022). One presumptive positive colony from each plate was inoculated into 10 mL of Tryptic Soy Broth (TSB; BD Diagnostics, Franklin Lakes, NJ), incubated for 18-24 hours at 37˚C, and confirmed for *Salmonella* using BAX® *Salmonella* Real-time PCR Assay with a 5 µl aliquot. Then, 1 ml aliquots with 10% glycerol concentration were saved in a -80˚C freezer for future serotyping.

For agar plates with uncharacteristic *Salmonella* growth or no growth, automatic immunomagnetic separation was performed on the LNH for the respective sample using anti-*Salmonella* beads (Dynabeads; Thermo Sci or Invitrogen, Carlsbad, CA) and a KingFisher™ mL Purification System (Thermo Scientific, Waltham, MA) as described by Miller et al. (2022). The beads (~110 µl) were then transferred into 3 mL of Rappaport-Vassiliadis (Remel, St. Louis, MO) broth, incubated for 18-20 hours at 42˚C, streaked for isolation onto XLD agar plates, and

incubated for 18-24 hours at 37˚C. Plates that contained characteristic *Salmonella* colonies (black colonies) were streaked onto XLD and frozen as described above.

3.2.6 Data processing

Detection of *Salmonella* was carried out using the BAX® System at 24h enrichment. The limit of detection (LOD) was 0 $\log_{10}$ (CFU/Sample) (i.e., 1 CFU/Sample). Each carcass contributed 5~7 sample types and was considered *Salmonella* positive if any of its sampled type was positive. In addition, the number of *Salmonella* positive sample types was tallied for each carcass.

Quantification of *Salmonella* was done using BAX®-System-SalQuant® and XLD at 6h enrichment. The limit of quantification (LOQ) was 1 $\log_{10}$(CFU/Sample) (i.e., 10 CFU/Sample) for BAX®-System-SalQuant® and 0.5 CFU/mL for XLD. The following Table 3-1 specifies how the *Salmonella* CFU/Sample (enumerated by BAX®-System-SalQuant®) were reported in various situations.

Table 3-1. Explanatory variables in a model representing the level of *Salmonella* contamination in a sample (detected and quantified by BAX®-System-SalQuant® were categorized into three groups: ND; [LOD, LOQ); ≥LOQ.

24h Enrichment Detection	6h Enrichment Quantification	CFU/Sample
Neg.	Not Available	Non-Detect (ND)
Pos.	<LOQ	[1,10)
Pos.	≥LOQ	Value derived from the standard curve

3.2.7 Statistical analysis for prevalence, enumeration, and method comparison

Data were analyzed by Dr. Qing Kang of the Kansas State University Statistical Consulting Center using the methods described. All tests were conducted at the 0.05 significance level. Comparisons between two levels of a fixed effect were carried out using two-sided tests. Statistical analyses were performed using the Statistical Analysis Software (SAS 9.4; Cary, NC).

The *Salmonella* prevalence status data was analyzed separately, for mesenteric LN, tonsils, and carcass (respective 35%, 36% and 62% overall prevalence rate), using the logit linear mixed model. Fixed effects of the model included season, region, and their interaction. The random effect was abattoir visit nested within season and region. Distributions of test statistics were approximated by Chi-square distributions. P-values were obtained via the Wald test. For mesenteric LN, the season-by-region interaction was not significant, and the final model contained main effects only. Fixed effects were evaluated in terms of estimated prevalence rates,

their 95% Wald confidence intervals, and odds ratios. Statistical analyses were performed using the SAS GLIMMIX procedure.

The *Salmonella* prevalence status data for axillary, inguinal, subiliac, tracheobronchial, and pre-scapular LNs all had overall prevalence rates ≤10%. They were first analyzed by the logit linear model with season and region being the fixed effects. The random effect of abattoir was ignored to assure model convergence and stability. For axillary and tracheobronchial LNs, the deviance/likelihood-ratio test indicated that the season-by-region interaction was significant and the model with main effects only had poor fitness; p-values were then obtained via the Fisher's exact test; the season-by-region effect was evaluated in terms of estimated prevalence rates, their 95% Pearson-Clopper confidence intervals, and odds ratios. For inguinal, subiliac, and pre-scapular LNs, the model with main effects only was adequate and p-values were obtained via the likelihood ratio test; main effects were evaluated in terms of estimated prevalence rates, their 95% Wald confidence interval, and odds ratios. Statistical analyses were performed using the SAS GENMOD and FREQ procedures.

The *Salmonella* enumeration data (quantified by BAX®-System-SalQuant®) was analyzed only for mesenteric LN and tonsils samples that were *Salmonella* positive. Portions of mesenteric LN samples (110 out of 208) and tonsil samples (88 out of 212) fell between the LOD and the LOQ. To account for samples that fell below LOQ, the lognormal accelerated failure time model (i.e., a parametric model for the left-censored data) was employed (Gillespie et al., 2010; Klein & Moeschberger, 2006). Fixed effects of the model included season, region, and their interaction. The random effect of abattoir was ignored. P-values were obtained via the Wald test; interactions were significant and were evaluated in terms of $\log_{10}$(CFU/Sample) least

squares means (LSM), their 95% Wald confidence intervals, and difference in LSMs. Statistical analyses were performed using the SAS LIFEREG procedure.

The number of *Salmonella* positive samples per carcass was an ordinal outcome. Data from the 528 carcasses with all 7 sample types collected was analyzed by the cumulative logit linear mixed model. Fixed effects included season, region, and their interaction. The random effect was abattoir visit nested within season and region. Distributions of test statistics were approximated by Chi-square distributions. P-values were obtained via the Wald test. Fixed effects were evaluated in terms of estimated rate for number of positive samples and the cumulative odds ratios for having fewer positive samples. Statistical analyses were performed using the SAS GLIMMIX procedure.

The receiver operating characteristic (ROC) curve plots sensitivity vs. one minus specificity of a model and was used to determine if *Salmonella* levels in various sample types could serve as the sentinel for *Salmonella* prevalence in the mesenteric LN and tonsils. Specifically, the *Salmonella* prevalence status in the mesenteric LN of a carcass was modelled using the logistic regression with explanatory variables being *Salmonella* levels (either ND, [LOD, LOQ), or ≥LOQ) of the other six sample types from the same carcass; the *Salmonella* prevalence status in tonsils of a carcass was modelled by the *Salmonella* levels of the other six sample types from the same carcass. Hosmer and Lemeshow provided the guideline for the interpreting area under the curve (AUC) of the ROC function as: AUC<0.6, failed prediction; 0.6≤AUC<0.7, poor prediction; 0.7≤AUC<0.8, acceptable prediction; 0.8≤AUC<0.9, excellent prediction; AUC≥0.9, outstanding prediction (Hosmer et al., 2013). Data from the 528 carcasses with all 7 sample types collected was analyzed using the SAS LOGISTIC procedure.

The BAX®-System-SalQuant® and XLD methods (with 6h enrichment) were compared based on *Salmonella* positive samples (detected by BAX®-System-SalQuant® with 24h enrichment; tonsils were excluded; n=378). The Cohen's kappa statistic was used to measure the degree of agreement, in terms of the ability to quantify *Salmonella*, between the two methods. Landis and Koch assigned the following benchmarks for agreement strength: poor (<0.00), slight (0-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) and almost perfect (0.81-1.00) (Landis & Koch, 1977). Here kappa was 0.37 with the 95% confidence interval of (0.27, 0.46), suggesting fair/moderate agreement. For samples quantifiable by both methods, the mean $\log_{10}$(CFU/Sample) difference was evaluated using the one-sample *t*-test for non-zero difference and one-sample confidence interval. Statistical analyses were performed using the SAS FREQ and MEANS procedures.

3.3 Results and discussion

3.3.1 Prevalence and enumeration

A total of 4,132 samples were collected in this study; 590 samples were positive for *Salmonella* when evaluated using the BAX *Salmonella* Real Time PCR Assay detection method. The mesenteric LNs and the tonsils had the highest prevalence, at 35% (n=600) and 36% (n=591), respectively. The market hog carcasses had a 62% (n=601) prevalence for *Salmonella*. The overall LN prevalence of the study was 14% (n=4,132), with the highest overall prevalence occurring during the spring season (17% (n=1,399)), in comparison to 14% (n=1,359) and 12% (n=1,374) during winter and fall, respectively. The overall prevalence of *Salmonella* among all sample types was 14% in this study, which is lower than the 21.8% prevalence (samples included mesenteric, inguinal, subiliac, and tracheobronchial LNs) reported by Miller et al. (2022) and the

27.7% (sample types included ileum, ileocolic and mandibular LNs, and tonsils) reported by Vieira-Pinto et al. (2005). In the present study, tonsils had the greatest overall prevalence of *Salmonella*, at 36%, whereas mesenteric LN represented the largest prevalence in studies by Vieira-Pinto et al. (2005) and Miller et al. (2022) at 35% and 34%, respectively. In comparison, Chaves et al. (2017) reported that mesenteric LNs had a 44% and 20% prevalence in two regions in Mexico, whereas the tonsils had an 18% and 40% prevalence in the two regions (Chaves et al., 2017).

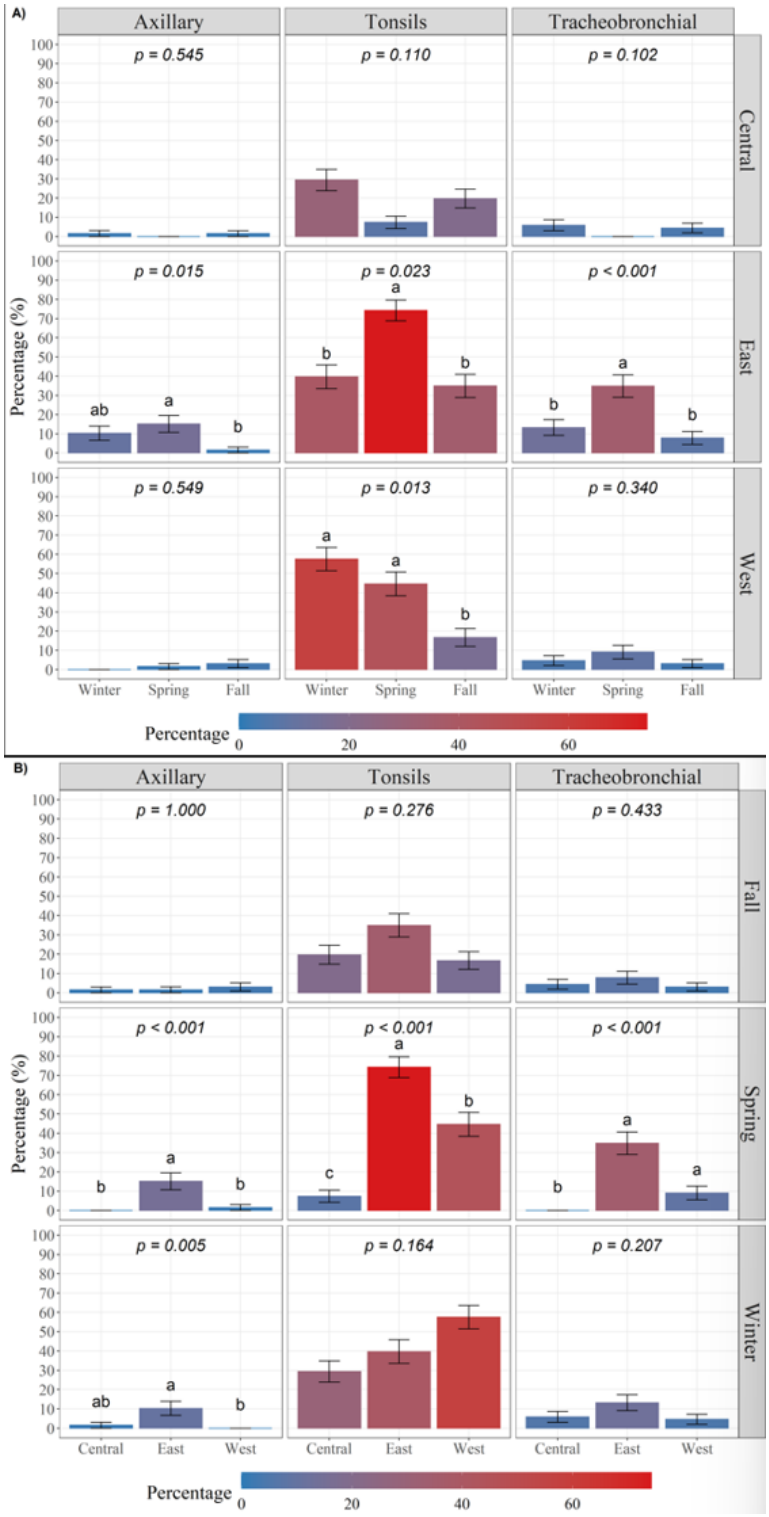

Figure 3-1. Graphical representation of *Salmonella* prevalence (%) by sample type with corresponding p-values where the season-by-region interaction was significant for prevalence (axillary LNs (*P*=0.038), tonsils (*P*=0.006), and tracheobronchial LNs (P=0.007)). The figure is divided by (A) *Salmonella* prevalence by region (central, east, and west) and (B) *Salmonella* prevalence by season (winter, spring, and fall) for the three significant (*P*<0.05) sample types.

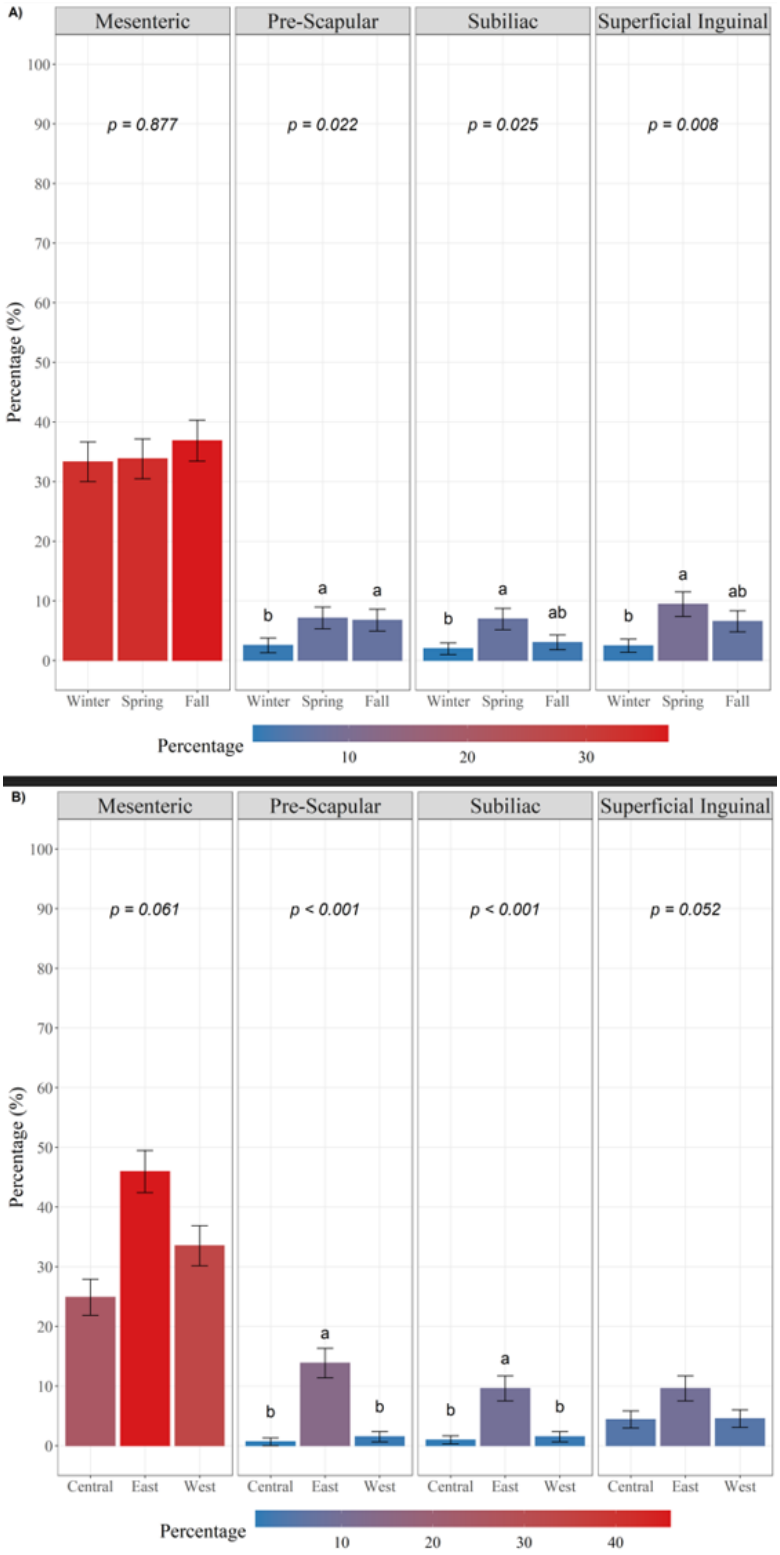

Figure 3-2. Graphical representation of *Salmonella* prevalence (%) by sample type with corresponding p-values where the season-by-region interaction was not significant for prevalence (mesenteric LNs (*P*=0.188), pre-scapular LNs (*P*=0.157), subiliac LNs (*P*=0.136), and superficial inguinal LNs (*P*=0.122)). The figure is divided by (A) *Salmonella* prevalence by season (winter, spring, and fall) for each sample type and (B) *Salmonella* prevalence by region for each sample type (central, east, and west) for the four sample types that were not significant (*P*>0.05) for season-by-region interaction.

A season-by-region interaction was detected for *Salmonella* prevalence of axillary LN (P=0.038), tracheobronchial LN (P=0.007) and tonsils (P=0.006) as shown in Figure 3-1. Figure 3-1A displays the data with region on the y-axis, while Figure 3-1B displays the data with season on the y-axis. Among these three sample types, tonsils had the highest prevalence, with the greatest prevalence observed in the eastern region during the spring season (74.2% (n=132)). While tonsils consistently had the highest prevalence during each season when compared to axillary and tracheobronchial LNs, it is important to point out that LN types were not statistically compared to one another, and this is a general observation.

As shown in Figure 3-1A, tonsils and the axillary and tracheobronchial LNs varied by season within the eastern region, with prevalence significantly higher ($P \leq 0.05$) in spring for tonsils (74.2%) and tracheobronchial LNs (34.8%). Axillary LN prevalence in the eastern region was highest in spring (15.2%), which was significantly higher (P=0.009) than prevalence in fall (1.6%). Tonsils were the only sample type that varied by season in the western region, with winter (57.6%) and spring (44.6%) prevalence both significantly higher ($P \leq 0.05$) than fall (15.8%). These same general trends can be observed in Figure 3-1B, which illustrates regional variation within season for tonsils and the axillary and tracheobronchial LNs. However, Figure 3-1B also highlights that axillary prevalence is highest in the east during winter (10.3%), which is greater than (P=0.013) the 0.0% prevalence in the western region, but statistically the same (P=0.063) as the 1.6% prevalence reported in the central region.

Table 3-2. Summary of *Salmonella* prevalence status reported by carcass in each season and region. Season by region interaction for *Salmonella* prevalence was significant for carcass (*P*=0.001).

Season	Region	Prevalence		
		Carcass		
		# of Carcasses	Count	Rate
Winter	**West**	66	44	66.7%
	Central	68	38	55.9%
	East	68	52	76.5%
	Across	202	134	66.3%
Spring	**West**	66	43	65.2%
	Central	69	18	26.1%
	East	66	60	90.9%
	Across	201	121	60.2%
Fall	**West**	66	39	59.1%
	Central	68	35	51.5%
	East	64	41	64.1%
	Across	198	115	58.1%
All		601	370	61.6%

A season-by-region interaction was also observed (*P*=0.001) for *Salmonella* prevalence at the carcass-level. Percent prevalence data for carcasses are displayed in Table 3-2. In the central region, carcass prevalence varied by season (*P*=0.007) with prevalence significantly higher ($P \leq 0.05$) in winter (55.9%) and fall (51.5%) in comparison to spring (26.0%). In the eastern region, carcass prevalence was significantly higher in spring (90.9%) than winter (76.5%; *P*=0.048) or fall (64.2%; *P*=0.002). In the spring season, *Salmonella* prevalence in market hog carcasses varied by region ($P<0.001$) as follows: 90.9% in the east, 65.2% in the west, and 26.0% in central, and all regions were significantly different than one another ($P \leq 0.05$).The main

effect of season was not significant for mesenteric LNs (P=0.877) but was significant for superficial inguinal (P=0.008), subiliac (P=0.025), and pre-scapular (P=0.022) LNs (Figure 3-2A). In general, mesenteric LNs had the highest prevalence (Figure 3-2A) at 31.7%, 32.6%, and 36.2% during winter, spring, and fall, respectively. Pre-scapular LN prevalence was lowest in winter (0.8%) when compared to spring (3.2%; P=0.011) and fall (3.0%; P=0.018). The spring subiliac LN prevalence (4.4%) significantly higher (P=0.010) than winter (1.1%). A similar trend was observed for superficial inguinal LN prevalence significantly greater (P=0.002) in spring (8.9%) in comparison to winter (2.3%). Little is known about the seasonal variability of *Salmonella* in market hog lymph nodes. A seasonal effect has been observed for *Salmonella* prevalence in beef LN studies; prevalence was significantly higher (P=0.0304) in the summer/fall months when compared to the winter/spring months (Gragg et al., 2013), which is consistent with the occurrence of human salmonellosis, as the CDC reports cases are more common during the summer months than in the winter (CDC, 2019).

The main effect of region was not significant for mesenteric (P=0.061) or superficial inguinal (P=0.052) LNs but was significant for subiliac (P<0.001) and pre-scapular (P<0.001) LNs (Figure 3-2B). Prevalence in the eastern region was significantly higher ($P\leq$0.05) for both subiliac (8.5%) and pre-scapular (12.4%) LNs when compared to prevalence in the central and western regions for both LN types. Although LN types were not statistically compared, it should be noted that mesenteric lymph node prevalence was also the highest in each region at 45.6%, 32.5%, and 23.9% in the eastern, western, and central regions, respectively. A study conducted by Bessire et al. (2018) reported a significant difference in *Salmonella* prevalence among sow inguinal LNs between the northern and southern regions (Bessire et al., 2018); though, it is difficult to compare location between these two studies because regions are defined differently.

Sows in the northern region had a higher occurrence of *Salmonella*. It is also important to consider that *Salmonella* prevalence likely varies between market hogs (present study) and sows (Bessire et al., 2018). In a similar beef LN study, it was reported that states located in the southern part of the U.S. had significantly higher (*P*=0.0198*) Salmonella* prevalence in subiliac lymph nodes from feedlot cattle than the northern region (Gragg et al., 2013).

The goal of sampling six types of LNs and the tonsils was to aid in market hog carcass mapping of *Salmonella* contamination. Mesenteric and tracheobronchial LNs were sampled to represent contamination within the viscera. Peripheral LNs (such as subiliac and superficial inguinal) were sampled to represent the LN types at risk of being incorporated into final meat products (Miller et al., 2022). Tonsils were used as a marker for contamination by the oral route, including while in lairage and possibly from shedding during transportation (Chaves et al., 2017; Hurd et al., 2001). *Salmonella* was found in each sample type in this study; however, *Salmonella* was not observed in all seven sample types from a single carcass, as shown in Table 3-3. Table 3-3 describes the number of carcasses containing between 0 and 7 positive samples. *Salmonella* was not detected in 38% of carcasses; it was detected in at least one LN in 36% of carcasses, and at least 2 LNs in 17% of carcasses. For the sake of simplicity, tonsils are included in the LN count in Table 3-3. Figure 3-3 displays the number of positive sample types by carcass in each season and region, providing another way to view the data provided in Table 3-3. In general, spring in the eastern region had more positive sample types from a carcass (i.e., the most frequently observable number of 6 positive sample types from one carcass). Miller et al. (2022) analyzed the prevalence data in a similar way to Table 3-3 below (sample types included mesenteric, inguinal, subiliac, and tracheobronchial LNs, all of which were sampled in this current study). *Salmonella* was not detected in 38% of the carcasses, but none of the carcasses

contained *Salmonella* in all four LN sample types. Overall, 40% of the carcasses had *Salmonella* in one LN type (Miller et al., 2022). The results described by Miller et al. (2022) are very similar to the results displayed in Table 3-3, where *Salmonella* was not detected in any LN for 38% of the carcasses in both studies. Moreover, in both Miller et al. (2022) and the present study, *Salmonella* was not observed in all four or seven sample types from a single carcass. These data may suggest that there are certain LN types that are more frequently positive for *Salmonella* and therefore pose a greater risk to contamination of consumer products.

Table 3-3. Distribution of *Salmonella* in lymph nodes by market hog carcass. Tonsils are included in the lymph node count.

Number of Lymph Nodes Harboring *Salmonella*	Number of Carcasses (%)
0	38.40% (231/601)
1	36.60% (220/601)
2	17.10% (103/601)
3	5.49% (7/601)
4	1.16% (7/601)
5	0.83% (5/601)
6	0.33 (2/601)
7	0.00% (0/601)

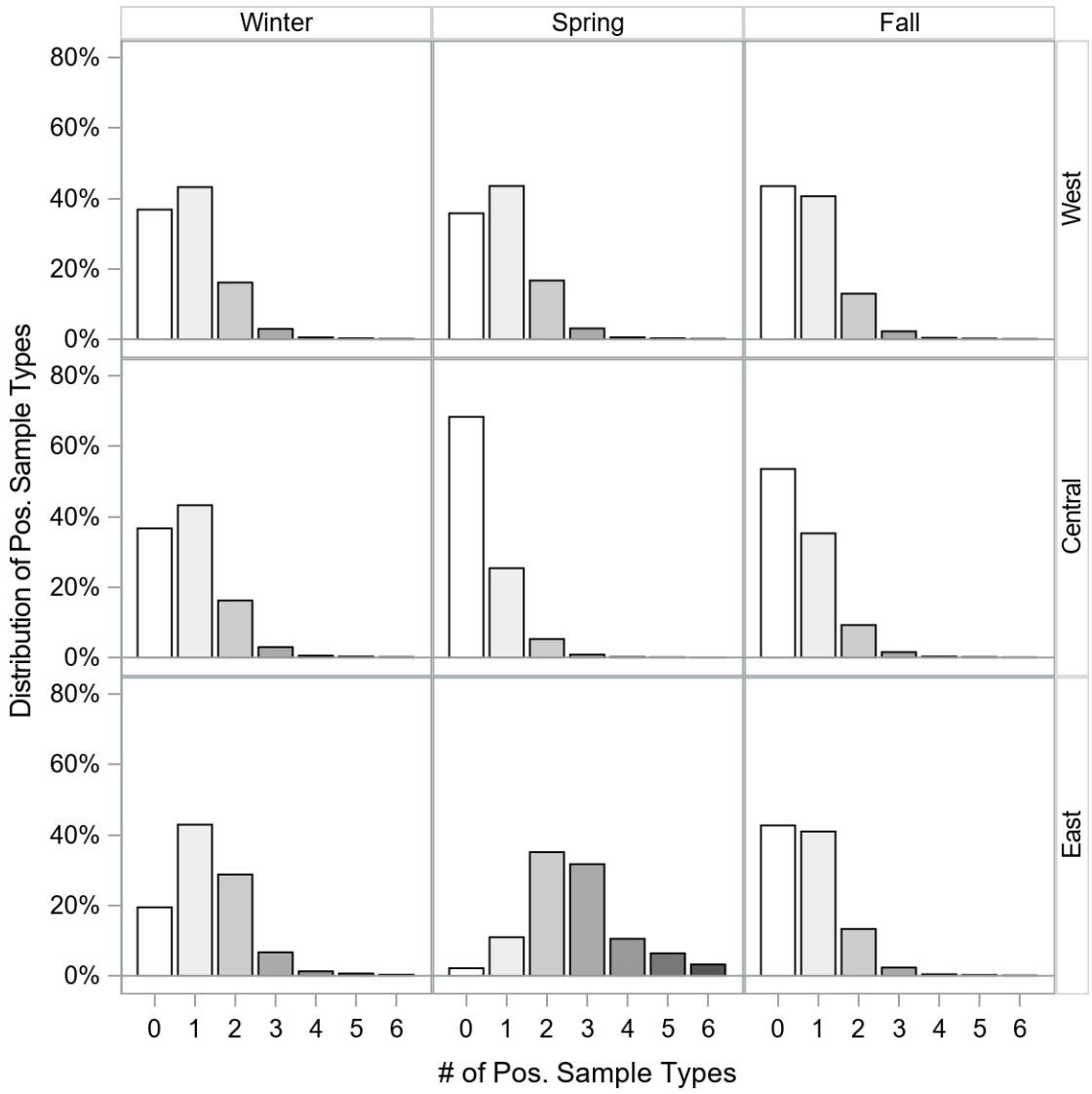

Figure 3-3. Model-based estimate of the distribution of the number of *Salmonella* positive sample types by season and region.

A complete set of all seven samples (six LNs and tonsils) was collected from a total of 528 carcasses throughout this study. The *Salmonella* prevalence for these 528 carcasses was analyzed according to number of samples positive per carcass, and a season-by-region interaction (P=0.034) was observed. In the eastern region, carcasses sampled in the winter and fall were more likely ($P \leq 0.05$) to have a fewer number of samples positive for *Salmonella* than

carcasses sampled in the spring. During the spring season, all regions were significantly different ($P \leq 0.05$), with more positive samples collected from carcasses sampled in the east, followed by the west, and then central.

Excluding tonsils, 378 LNs were positive for *Salmonella*, of which 150 were quantifiable using BAX®-System-SalQuant® while the other 228 samples fell below the LOQ (meaning the samples were positive for prevalence, but not able to be quantified). Enumeration median values varied depending on sample type; not all sample types were able to be quantified, with all median *Salmonella* enumeration values falling below the limit of quantification, except for tonsils. The *Salmonella* enumeration data were analyzed only for mesenteric LN and tonsils samples that were *Salmonella* positive because these were the only sample types with enough positive samples at concentrations above the limit of quantification to statistically analyze. Several mesenteric LN samples (110 out of 208) and tonsil samples (88 out of 212) harbored *Salmonella* at concentrations between the LOD and the LOQ.

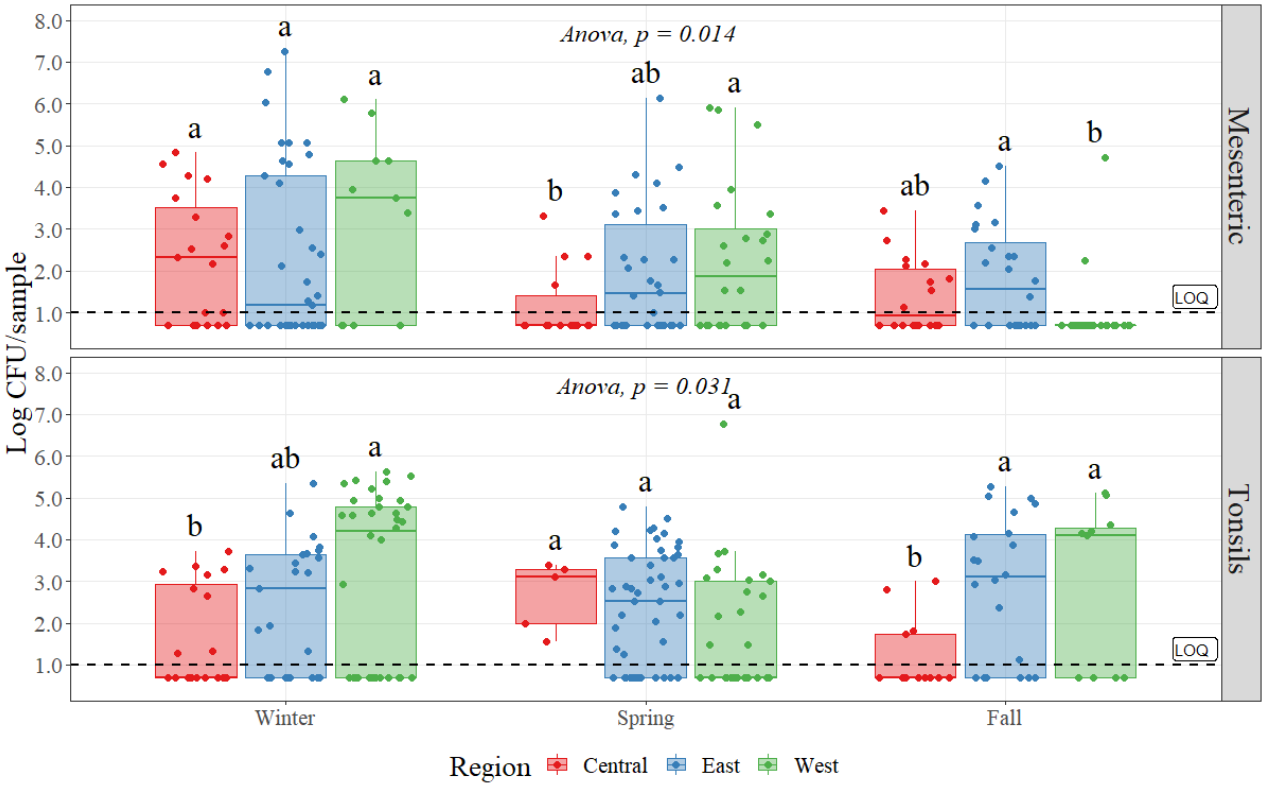

Figure 3-4. Bar charts of *Salmonella* $\log_{10}$ (CFU/Sample) enumeration from ANOVA test organized by region with season for sample types where season-by-region interaction was significant (mesenteric LNs (*P*=0.014) and tonsils (*P*=0.031) and were *Salmonella* positive. Only mesenteric LNs and tonsils were statistically analyzed for *Salmonella* enumeration using BAX®-System-SalQuant®.

A season-by-region interaction for *Salmonella* concentration in mesenteric LNs (*P*=0.014) and tonsils (*P*=0.031) was significant (Figure 3-4). In general, concentration of *Salmonella* appears to be the greatest during the winter season for mesenteric LNs, though concentration did not vary by region during this season (*P*>0.05). During the spring season, the *Salmonella* concentration in mesenteric LNs from the western region (2.2 $\log_{10}$ CFU/sample) was significantly greater (*P*=0.034) than in the central region (1.1 $\log_{10}$ CFU/sample). Mesenteric LN *Salmonella* concentration in the fall was significantly greater (*P*=0.013) in the eastern region (1.8 $\log_{10}$ CFU/sample) than in the western region (0.7 $\log_{10}$ CFU/sample). Median *Salmonella* concentration for mesenteric LNs fell below the limit of quantification for

BAX®-System-SalQuant®. *Salmonella* concentration in tonsils during the winter in the western region was 3.0 $\log_{10}$ CFU/sample, which was significantly greater (P=0.001) than the 1.5 $\log_{10}$ CFU/sample observed in the central region during the same season. *Salmonella* concentration in tonsils also varied during the fall season, with the eastern region (2.8 $\log_{10}$ CFU/sample) and western region (2.7 $\log_{10}$ CFU/sample) significantly greater (P>0.05) than the central region (1.1 $\log_{10}$ CFU/sample). The median *Salmonella* concentration for tonsils was 2.18 $\log_{10}$ CFU/sample.

Evaluation of *Salmonella* concentration in market hog lymph nodes is limited, especially in comparison to beef lymph nodes. Recently, *Salmonella* enumeration was explored in four LN types (mandibular, superficial inguinal, superficial popliteal, and medial iliac) where most enumeration values from positive samples were below 0.66 $\log_{10}$ MPN/g. However, the highest concentrations of *Salmonella* were 3.9 and 3.7 $\log_{10}$ MPN/g (Larsen et al., 2023). On the beef side, mesenteric LNs have been found to contain up to 5000 CFU/g of *Salmonella* (Samuel et al., 1980; Vargas et al., 2023). Moreover, another beef lymph node study reported a mean *Salmonella* concentration of 1.75 $\log_{10}$ CFU/g, with a range up to 3.8 $\log_{10}$ CFU/g (Gragg et al., 2013). Furthermore, peripheral beef LN *Salmonella* concentrations have been reported to range from 1.6 to 4.9 $\log_{10}$ CFU/LN (Webb et al., 2017). These studies use different enumeration methods, but all highlight the importance of exploring *Salmonella* concentration in LNs because they can contaminate comminuted product.

It is necessary to discuss the shortcomings of using the LN BAX®-System-SalQuant® curve to describe the tonsil results; as mentioned in the method comparison discussion above, tonsils harbored substantial background microflora that interfered with the 3M™ EB Petrifilm™ + XLD replica plate enumeration method. Because of this, a separate BAX®-System-SalQuant®

curve should be developed as a quantification method specifically for tonsils. Future research conducted on market hog tonsils should take this into account because the LN BAX®-System-SalQuant® quantification curve used in this study may not be an accurate method for tonsil quantification, and the concentration data presented herein for tonsils should be interpreted accordingly.

It is important to discuss that only mesenteric LNs and tonsils had enough positive samples with high *Salmonella* concentrations above the limit of quantification to be enumerated. Mesenteric LNs and tonsils aren't typically destined for comminuted pork and are, instead, disposed of at the plant, so these data are useful in understanding contamination within the gastrointestinal tract (Miller et al., 2022). The risk of *Salmonella* contamination of ground pork from these two sample types is limited in comparison to the peripheral LNs that are located in adipose tissue, and therefore risk to public health is limited as well.

3.3.2 Method comparison

One of the objectives of this study was to compare 3M™ EB Petrifilm™ + XLD replica plate method to BAX®-System-SalQuant®. Table 3-4 shows the number of samples that were *Salmonella* positive as determined by each methodology. Tonsils were not included in this comparison.

Table 3-4. Contingency table of the number of *Salmonella* positive samples quantified from market hog LNs using both methodologies of 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®.

BAX®-System-SalQuant®	3M™ EB Petrifilm™ + XLD replicate plate		Total
	< LOQ	Quantified	
< LOQ	183	45	228
Quantified	67	83	150
Total	250	128	378

[1]

[1] The lower limit of quantification for BAX®-System-SalQuant® is ~10 CFU/Sample (1 log CFU/Sample) for small LNs, and ~50 CFU/Sample (1.7 log CFU/Sample) for medium LNs. The lower limit of quantification for 3M™ EB Petrifilm™ + XLD replicate plate method is 0.5 CFU/mL (because petrifilms were plated in duplicate).

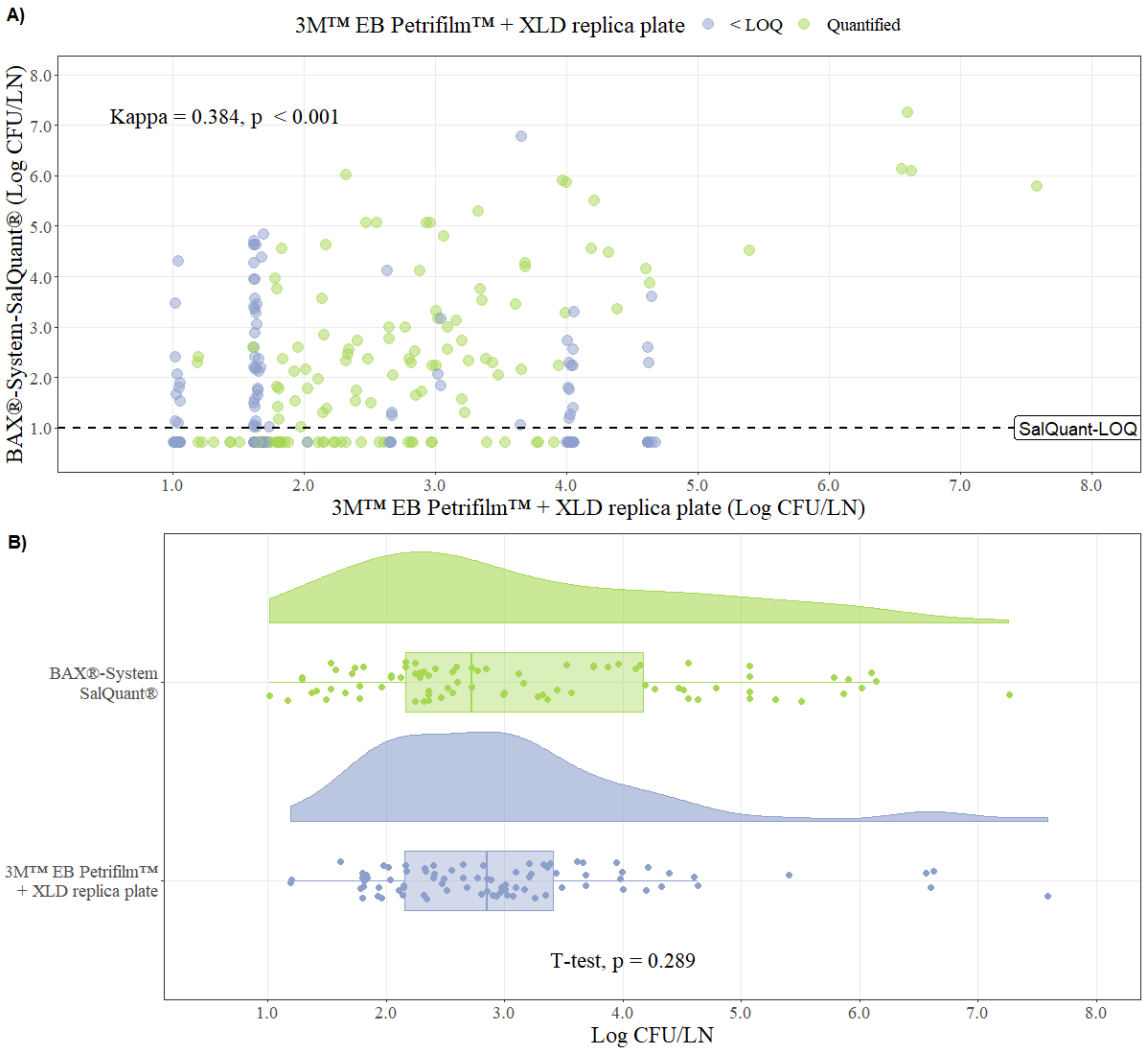

Figure 3-5. Graphical representation of the comparison between 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® for enumeration of *Salmonella* in pork lymph nodes. A) Enumeration of *Salmonella* (Log CFU/Sample) in pork lymph nodes using 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® (*n* = 378 samples). The dots represent the actual data points. LOQ: Limit of Quantification. B) Raincloud plot of *Salmonella* counts (Log CFU/Sample) using 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® in pork lymph nodes with counts above limit of quantification for both methodologies (*n* = 83 samples). In each boxplot, the horizontal line crossing the box represents the median, the bottom and top box are the lower and upper quartiles, the vertical top line represents 1.5 times the interquartile range, and the vertical bottom line represents 1.5 times the lower interquartile range. The dots represent the actual data points. Distribution plots were generated using the kernel density estimates as the probability density function of a continuous variable.

The contingency table (Table 3-4) displays the number of samples that were categorized from each methodology. The kappa coefficient between 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® is 0.384, which indicates a fair agreement between

the two methodologies. Of the 378 samples that were positive and quantifiable for *Salmonella*, 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® were both able to both quantify 83 samples out of 378 *Salmonella* positive samples. The 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® methods were both able to determine that 183 out of 378 samples lie below the limit of quantification. The 3M™ EB Petrifilm™ + XLD replica plate method quantified 45 samples that were not quantifiable by BAX®-System-SalQuant® and, conversely, the BAX®-System-SalQuant® quantified 67 samples that were not quantifiable by the 3M™ EB Petrifilm™ + XLD replica plate method. The blue dots on Figure 3-5A represent *Salmonella* positive samples that were quantifiable by BAX®-System-SalQuant® but were below the limit of quantification for 3M™ EB Petrifilm™ + XLD replicate plate method. The green dots represent positive samples that were quantifiable by both methodologies. The limit of quantification for 3M™ EB Petrifilm™ + XLD replicate plate is determined by sample weight and volume of media added for enrichment. BAX®-System-SalQuant® has a lower limit of quantification, which is 1 log CFU/Sample, or 10 CFU/Sample. The blue dots located on the left side of the graph in Figure 3-5A correspond with the limit of quantification that was described by Vargas et al. (2023) . For 3M™ EB Petrifilm™ + XLD replicate plate, the lowest value that can be estimated is 1 CFU/2 mL (0.5 CFU/mL) because of duplicate plating. For small LNs (<3 grams), 20 ml of BAX MP media was used to enrich with a sample weight on average being 2 grams, which would result in ~10 CFU/Sample lower limit (0.5 CFU/g * 22 total g in sample bag = 11) for estimation. This same process can be used to determine the lower limit for medium sized LNs (>3 grams); for 80 ml of media and an average sample weight of 10 to 20 grams, the limit would be ~50 CFU/ml (0.5 CFU/g * 100 total g in sample bag = 50). The $\log_{10}$ conversion of these values is 1 log CFU/Sample and 1.7 log CFU/Sample.

Addressing the issues of background growth and dilutions were the largest disadvantages in this study when comparing 3M™ EB Petrifilm™ + XLD replicate plate to BAX®-System-SalQuant®. In instances where too many colonies to count were observed on 3M™ EB Petrifilm™, a dilution would be performed to obtain a countable range for *Salmonella*; however, *Salmonella* colonies were often not enumerable on XLD plates once a countable range was determined on the 3M™ EB Petrifilm™ plate. The EB Petrifilm™ plate enumerates all members of the Enterobacteriaceae family; thus, background growth on the 3M™ EB Petrifilm™ prevented proper quantification; however, these samples were still positive for *Salmonella* according to BAX®-System-SalQuant®. Another variable affecting the stacks of blue dots in Figure 3-5A is the dilution factor used to obtain a countable range on 3M™ EB Petrifilm™ + XLD replicate plate. The stack of blue dots located on the left side of Figure 3-5A would represent samples below the limit of quantification with dilution factors of 0 or 1. The stack of blue dots on the right side of the figure represent samples below the limit of quantification with dilutions of 2 or 3. The quantification values from samples that were diluted on the 3M™ EB Petrifilm™ shown in Figure 3-5A do not represent actual numbers, but rather these values have been extrapolated to represent quantification values when background flora limits the ability for proper enumeration. The methods used to calculate the level of *Salmonella* from a 3M™ EB Petrifilm™ plate that has been diluted estimates a number, meaning the corresponding blue dots are estimates that lie below the limit of quantification for the 3M™ EB Petrifilm™ + XLD replicate plate method.

In Figure 3-5B, the t-test conducted on the 83 samples that were quantifiable by both methods resulted in a p-value of 0.289, suggesting that there was no significant difference in the mean log CFU/sample for either method. The distribution curve in Figure 3-5B also shows that

most samples were enumerated between the ranges of 1 and 3 logs. Out of the roughly 4,000 samples collected, only 4 LN samples were quantified at a level above 6.5 logs of *Salmonella* using 3M™ EB Petrifilm™ + XLD replica plate method. According to Teunis et al. (2010), 4 logs of *Salmonella* is concentrated enough to cause a 50% chance of human illness (Teunis et al., 2010). However, LNs are not directly consumed, rather they are mixed into batch of ground pork (i.e., thousands of pounds of product), resulting in a dilution effect. It must be acknowledged that there won't be an even distribution of *Salmonella* contamination within the batches of ground pork, so the true dilution effect is unknown. The risk with ground pork contamination lies with the number of LNs that go into the grind, as well as the number of those LNs that are contaminated (because as we can see from the prevalence data, not all LNs harbor *Salmonella*). There are several factors of *Salmonella* that will determine the effect on human health such as serotype, virulence factors, and dosage of bacteria. The amount of *Salmonella* in a sample and/or batch of ground pork will determine the risk of human infection. Lower concentrations of *Salmonella* are not as large of a risk in comparison, but can still cause foodborne illness (Teunis et al., 2010).

The 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® method of quantification can be used to enumerate *Salmonella*, as the t-test for the samples that were quantified with both methods showed no significant difference in methodologies for quantification. Determining which method to use will vary depending on laboratory and protocol because the 3M™ EB Petrifilm™ + XLD replicate plate and BAX®-System-SalQuant® each have advantages and disadvantages. For the 3M™ EB Petrifilm™ + XLD replica plate method, approximately 2 days are required to obtain data. During this process, there is room for human error, such as when removing the aliquot from the sample bag, plating on the Petrifilm™ media,

during replica plating onto XLD plates, and counting colonies. Another disadvantage to 3M™ EB Petrifilm™ + XLD replica plate method is the counting of black colonies on the XLD plates; while XLD is a selective media most frequently used for EB and *Salmonella*, not all *Salmonella* will grow as black colonies, and not all black colonies are *Salmonella*. The 3M™ EB Petrifilm™ + XLD replica plate method performs well at enumerating higher logs of *Salmonella*, such as values greater than 3 logs, as described by Vargas et al. (2023). In comparison, the BAX®-System-SalQuant® method takes 6 hours for incubation, with 75 minutes post-incubation to receive results. The BAX®-System-SalQuant® method determines absence (negative) or presence (positive) of a gene in *Salmonella*, which is more reliable than counting black colonies on XLD plates. A disadvantage of using BAX®-System-SalQuant® may be the aliquot size; BAX®-System-SalQuant® uses 5 microliters for quantification, in comparison to 1 ml used for 3M™ EB Petrifilm™ + XLD replica plate method. Furthermore, there are differences in limits of quantification between the two methodologies. The BAX®-System-SalQuant® has a predetermined lower limit of 10 CFU, while 3M™ EB Petrifilm™ + XLD replica plate method has a higher limit of 10 to 50 CFU (depending on sample size). Vargas et al. (2023) displays the linear correlation of 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® *Salmonella* quantification (Vargas et al., 2023). On average, 3M™ EB Petrifilm™ + XLD replica plate method estimates 3 to 5 logs more accurately, while BAX®-System-SalQuant® estimates 1 to 3 logs of *Salmonella* more accurately, as described by Vargas et al. (2023).

There are samples that were similarly quantifiable by both methodologies when considering the green dots in Figure 3-5A. For example, there are a cluster of green dots surrounding 3 log enumeration for 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®. Another green dot that can be highlighted from the figure is a green

dot at 5.4 logs on 3M™ EB Petrifilm™ + XLD replica plate method axis, and 4.5 log on BAX®-System-SalQuant® axis. While these values are within a 1 log difference, we should expect the 3M™ EB Petrifilm™ + XLD replica plate enumeration to be more accurate (Vargas et al., 2023). Conversely, BAX®-System-SalQuant® enumerated one sample in Figure 3-5A to be 2.2 logs, while 3M™ EB Petrifilm™ + XLD replica plate method enumerated the same sample as 3.9; for this sample, we should expect the BAX®-System-SalQuant® quantification to be more accurate. These interpretations are drawn from the linear correlations that describe the strength of estimation between the two methodologies described by Vargas et al. (2023).

Tonsils were removed from the method comparison because 3M™ EB Petrifilm™ + XLD replica plate data was collected during only one season. The results of these data led researchers to abandon 3M™ EB Petrifilm™ + XLD replica plate method for tonsils in spring and fall season. The background growth from other EB colonies was interfering with proper enumeration on these plates. Perhaps the pork LN BAX®-System-SalQuant® curve is not the proper method to evaluate *Salmonella* enumeration. It is recommended that a curve specific for tonsils is developed and used for pork tonsil enumeration.

3.4 Conclusions

In conclusion, the 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® methodologies can both be used to quantify *Salmonella* in market hog LNs. *Salmonella* prevalence varies by LN type in different seasons and regions. Tonsils and mesenteric LNs were associated with the greatest overall *Salmonella* prevalence, at 36% and 35%, respectively. The highest prevalence was observed in tonsils during the spring in the eastern region. The other five sample types had a prevalence <10%, though prevalence was

generally greatest in the eastern region and during the summer or fall months. It is important to mention that tonsils and mesenteric LNs are not commonly associated with ground pork contamination because they are removed from the carcass. However, the question may be raised about cheek meat contamination from the tonsils, as well as other cross-contamination risks. Peripheral LNs, such as subiliac, prescapular, superficial inguinal, and axillary LNs, have a higher likelihood of being incorporated into ground pork because of their location in fat in the carcass. Tonsils and mesenteric LNs also had the highest concentration of *Salmonella.* The median *Salmonella* values for mesenteric LNs fell below the limit of quantification, but the median values for tonsils was 2.18 log10 CFU/Sample using BAX®-System-SalQuant®. While most samples that were positive for *Salmonella* harbored less than 3 logs, there were several samples harbored greater than 3 logs. The dilution effect that occurs when a LN is incorporated into ground pork must be considered; more research may need to be conducted to understand how one LN can contaminate hundreds of pounds of ground pork in a batch. Pork processing facilities can use these findings to create mitigation strategies designed for controlling *Salmonella* contamination in LNs, though further research may be necessary to identify the best mitigation strategies. *Salmonella* continues to be a problem in pork systems, prompting the study of tissues that harbor this pathogen. The results from this study reinforced that market hog LNs harbor *Salmonella* in various lymphoid tissue and the risk of *Salmonella* contaminated LNs is greatest when the interior of a LN is exposed. Lymph nodes are also commonly associated with ground pork products, thus representing a risk for disease in humans if the products are not cooked or handled properly.

References

Arthur, T. M., Brichta-Harhay, D. M., Bosilevac, J. M., Guerini, M. N., Kalchayanand, N., Wells, J. E., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2008). Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. *Journal of food protection*, *71*(8), 1685-1688. https://doi.org/10.4315/0362-028X-71.8.1685

Aryal, S. (2022). *Xylose Lysine Deoxycholate (XLD) agar- principle, uses, composition, preparation and colony characteristics*. https://microbiologyinfo.com/xylose-lysine-deoxycholate-xld-agar-principle-uses-composition-preparation-and-colony-characteristics/

Bessire, B. C., Thomas, M., Gehring, K. B., Savell, J. W., Griffin, D. B., Taylor, T. M., Mikel, W. B., Campbell, J. A., Arnold, A. N., & Scaria, J. (2018). National survey of *Salmonella* prevalence in lymph nodes of sows and market hogs. *Translational animal science*, *2*(4), 365-371. https://doi.org/10.1093/tas/txy072

Bueno López, R., Vargas, D. A., Jimenez, R. L., Casas, D. E., Miller, M. F., Brashears, M. M., & Sanchez-Plata, M. X. (2022). Quantitative bio-mapping of *Salmonella* and indicator organisms at different stages in a commercial pork processing facility. *Foods*, *11*(17), 2580. https://doi.org/10.3390/foods11172580

CDC. (2015, December 2, 2015). *Multidrug-resistant Salmonella I 4,[5],12:i:- and Salmonella Infantis infections linked to pork, 2015*. CDC. https://www.cdc.gov/salmonella/pork-08-15/index.html

CDC. (2019, February 8, 2019). *Prevention*. CDC. https://www.cdc.gov/salmonella/general/prevention.html#:~:text=Salmonella%20illness%20is%20more%20common,conditions%20for%20Salmonella%20to%20grow.

CDC. (2022a, December 30, 2022). *Previous outbreaks*. CDC. https://www.cdc.gov/salmonella/outbreaks.html

CDC. (2022b, September 9, 2022). *Questions & answers*. CDC. https://www.cdc.gov/salmonella/general/index.html#:~:text=How%20common%20is%20Salmonella%20infection,the%20United%20States%20every%20year.

Chaves, B. D., Ruiz, H., Garcia, L. G., Echeverry, A., Thompson, L., Miller, M. F., & Brashears, M. M. (2017). High prevalence of *Salmonella* in lymph nodes and tonsils of swine presented for slaughter in Mexico. *Food protection trends*, *37*(1), 25.

Giannella, R. A. (1996). *Medical microbiology* (S. Baron, Ed. 4th ed.) https://www.ncbi.nlm.nih.gov/books/NBK8435/

Gillespie, B. W., Chen, Q., Reichert, H., Franzblau, A., Hedgeman, E., Lepkowski, J., Adriaens, P., Demond, A., Luksemburg, W., & Garabrant, D. H. (2010). Estimating population distributions when some data are below a limit of detection by using a reverse Kaplan-Meier estimator. *Epidemiology (Cambridge, Mass.)*, *21*(4), S64-S70. https://doi.org/10.1097/EDE.0b013e3181ce9f08

Gragg, S. E., Loneragan, G. H., Brashears, M. M., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Wang, R., Schmidt, J. W., Brooks, J. C., Shackelford, S. D., Wheeler, T. L., Brown, T. R., Edrington, T. S., & Brichta-Harhay, D. M. (2013). Cross-sectional

study examining *Salmonella* enterica carriage in subiliac lymph nodes of cull and feedlot cattle at harvest. *Foodborne pathogens and disease*, *10*(4), 368-374. https://doi.org/10.1089/fpd.2012.1275

Hosmer, D. W., Lemeshow, S., & Sturdivant, R. X. (2013). *Applied logistic regression, third edition* (3rd ed.). John Wiley and Sons. https://doi.org/10.1002/9781118548387

Hurd, H. S., McKean, J. D., Wesley, I. V., & Karriker, L. A. (2001). The Effect of lairage on *Salmonella* isolation from market swine. *Journal of food protection*, *64*(7), 939-944. https://doi.org/10.4315/0362-028X-64.7.939

Klein, J. P., & Moeschberger, M. L. (2006). *Survival analysis: techniques for censored and truncated data* (Second Edition. ed.). Springer. https://doi.org/10.1007/b97377

Landis, J. R., & Koch, G. G. (1977). A one-way components of variance model for categorical data. *Biometrics*, *33*(4), 671-679. https://doi.org/10.2307/2529465

Larsen, S., Bokenkroger, C., Delago, J., & Radtke, D. (2023, 15 May 2023). *Salmonella enterica prevalence and serotype distribution in lymph nodes from market swine* SafePork 2023, New Orleans, LA. https://www.iastatedigitalpress.com/safepork/article/id/16359/

Lei, S., Chen, S., & Zhong, Q. (2021). Digital PCR for accurate quantification of pathogens: principles, applications, challenges and future prospects. *International journal of biological macromolecules*, *184*, 750-759. https://doi.org/10.1016/j.ijbiomac.2021.06.132

Mann, E., Wagner, M., Schmoll, F., Slaghuis, J., Schönenbrücher, H., & Mester, P. (2014). Rapid testing and quantification of *Salmonella* in ileocaecal lymph nodes of Austrian pigs

slaughtered for consumption. *Research in veterinary science*, *97*(2), 187-190. https://doi.org/10.1016/j.rvsc.2014.06.016

Matthews, K. R., Kniel, K. E., & Montville, T. J. (2017). *Food microbiology- an introduction (4th Edition)* (4th ed.). American Society for Microbiology (ASM).

Miller, M., Maher, J. M., Wiseman, B., & Gragg, S. E. (2022). *Salmonella* is present in multiple lymph nodes of market hog carcasses at slaughter. *Food protection trends*, *42*(2), 100-106. https://doi.org/https://doi.org/10.4315/FPT-21-027

Samuel, J. L., O'Boyle, D. A., Mathers, W. J., & Frost, A. J. (1980). Distribution of *Salmonella* in the carcases of normal cattle at slaughter. *Research in veterinary science*, *28*(3), 368-372. https://doi.org/10.1016/S0034-5288(18)32724-3

Self, J. L., Luna-Gierke, R. E., Fothergill, A., Holt, K. G., & Vieira, A. R. (2017). Outbreaks attributed to pork in the United States, 1998-2015. *Epidemiology & Infection*, *145*(14), 2980-2990. https://doi.org/10.1017/s0950268817002114

Shahbandeh, M. (2022a, November 25, 2022). *Meat consumption worldwide from 1990 to 2021, by meat type**. Statista. https://www.statista.com/statistics/274522/global-per-capita-consumption-of-meat/

Shahbandeh, M. (2022b, December 16, 2022). *Per capita meat consumption in the United States in 2021 and 2031, by type*. Statista. https://www.statista.com/statistics/189222/average-meat-consumption-in-the-us-by-sort/#:~:text=In%202021%2C%20the%20most%20consumed,pounds%20per%20capita%20by%202031.

Shahbandeh, M. (2022c, April 13, 2022). *Production of pork worldwide from 2013 to 2022 (in million metric tons)***. Statista. https://www.statista.com/statistics/237570/pork-production-worldwide/

Shahbandeh, M. (2022d, July 18, 2022). *Projected pig meat consumption worldwide from 2021 to 2031 (in 1,000 metric kilotons)**. Statista. https://www.statista.com/statistics/739879/pork-consumption-worldwide/

Teunis, P. F. M., Kasuga, F., Fazil, A., Ogden, I. D., Rotariu, O., & Strachan, N. J. C. (2010). Dose–response modeling of *Salmonella* using outbreak data. *International journal of food microbiology*, *144*(2), 243-249. https://doi.org/10.1016/j.ijfoodmicro.2010.09.026

Vargas, D. A., Betancourt-Barszcz, G. K., Blandon, S. E., Applegate, S. F., Brashears, M. M., Miller, M. F., Gragg, S. E., & Sanchez-Plata, M. X. (2023). Rapid quantitative method development for beef and pork lymph nodes using BAX ® system real time *Salmonella* Assay. *Foods*, *12*(4).

Webb, H. E., Brichta-Harhay, D. M., Brashears, M. M., Nightingale, K. K., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Schmidt, J. W., Wang, R., Granier, S. A., Brown, T. R., Edrington, T. S., Shackelford, S. D., Wheeler, T. L., & Loneragan, G. H. (2017). *Salmonella* in peripheral lymph nodes of healthy cattle at slaughter. *Frontiers in microbiology*, *8*, 2214-2214. https://doi.org/10.3389/fmicb.2017.02214

WHO. (2022, May 19, 2022). *Food safety*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/food-

safety#:~:text=Salmonella%2C%20Campylobacter%20and%20enterohaemorrhagic%20Escherichia,with%20severe%20and%20fatal%20outcomes.

Chapter 4 - Serotyping of *Salmonella* Isolated from Market Hog Lymph Nodes and Tonsils

Abstract

Salmonella is one of the most important pathogens that cause foodborne disease, resulting in millions of foodborne illnesses around the world. *Salmonella* infections can frequently be caused by contaminated food products, and livestock are a natural reservoir for this bacterium. There are thousands of serotypes of *Salmonella*, however not all are pathogenic to humans. Serotypes are an important indicator of the risk of pathogenesis to people. This study investigated the *Salmonella* serotypes isolated from market hog lymph nodes (LNs) and tonsils. Lymphoid tissues can harbor pathogens such as *Salmonella*. Contaminated lymph nodes that are sliced open or ground into comminuted pork are a food safety risk. *Salmonella* isolates recovered were serotyped to evaluate risk. The most frequently isolated serotype was monophasic *S.* Typhimurium, followed by *S.* Agona and *S.* Schwarzengrund, accounting for 25%, 16%, and 11% of the isolates, respectively.

4.1 Introduction

Salmonella is one of the leading causes of foodborne illness in the world (WHO, 2022). Researchers estimate that *Salmonella* will infect more than 1.3 million people each year in the United States, and more than 93 million people each year in the world (CDC, 2023; Gong et al., 2022). Reservoirs include the gastrointestinal tract of humans and livestock, including hogs (Gut

et al., 2018). *Salmonella* is mesophilic, Gram-negative, rod-shaped, non-spore forming, and is motile by peritrichous flagella (Giannella, 1996; Gut et al., 2018; Matthews et al., 2017).

Serotypes of *Salmonella* are determined by the antigens found on the surface of the bacterial cell. Currently, there are greater than 2,500 serotypes that have been identified (CDC, 2022b; Ferrari et al., 2019). *Salmonella enterica* and *S. bongori* are the two species of *Salmonella*, with the former containing the most serotypes and causing most human infections (Ferrari et al., 2019). *S. enterica* is comprised of six subspecies: *S. enterica* subspecies (I) *enterica*, (II) *salamae*, (IIIa) *arizonae*, (IIIb) *diarizonae*, (IV) *houtenae*, and (VI) *indica*. Clinical isolates are most frequently associated with *S. enterica* subsp. *enterica* (Herrera-León et al., 2007), with more than half of the greater than 2,500 serotypes of *Salmonella* belonging to *S. enterica* subsp. *enterica* (Eng et al., 2015). The lipopolysaccharide layer characterizes the somatic O-antigen. Specific to *S. enterica* subsp. *enterica,* the most common O-antigens are A, B, C1, C2, D, and E (Brenner et al., 2000). The H-antigen is the flagellar antigen and can appear in phase 1 or phase 2 (Giannella, 1996). The phase is determined by the expression of the *fliC* or *fliB* gene, respectively (McQuiston et al., 2011). A third antigen, called the Vi-antigen, is a capsular polysaccharide antigen that contributes to the pathogenicity of the serotype, and is only present for a handful of serotypes (Giannella, 1996; Wain et al., 2005). *Salmonella* Typhi, *S.* Paratyphi C, and *S.* Dublin contain the Vi antigen (Matthews et al., 2017). *Salmonella* can be categorized as typhoidal or nontyphoidal strains (NTS). Typhoidal strains cause enteric fever like typhoid or paratyphoid fever (Gal-Mor et al., 2014), and NTS serotypes cause salmonellosis and are commonly associated with agricultural products (Acheson & Hohmann, 2001).

Virulence factors will determine the pathogenicity of *Salmonella* serotypes (Giannella, 1996). Serotype and pathogen dosage are other factors that will determine the severity of disease

in a human (Matthews et al., 2017). Host factors will also determine the severity of disease in humans; these factors include age and immune status (Giannella, 1996). Several studies have evaluated the amount *Salmonella* that is required to establish infection—the estimated range is 3 to 6 logs of *Salmonella*, depending on the serotype ingested (Canada, 2010; Gut et al., 2018; Teunis et al., 2010). *Salmonella* can be picked up by the lymphatic system of livestock, which is why this pathogen can be found in lymph nodes and tonsils of market hogs (Bonardi, 2017; Giannella, 1996). This pathogen can be carried asymptomatically by hogs in tonsils, lymphoid tissue, and gastrointestinal tract (Bonardi, 2017). Hogs have a curious nature and like to chew on things; this can spread pathogens from hog to hog during transportation or lairage time, especially between hogs from separate houses that are commingled and chew on lairage ropes (Bonardi, 2017; Hurd et al., 2001).

Serotyping of *Salmonella* is important for diagnostic and therapeutic purposes, as well as for tracking of foodborne disease outbreaks (Brenner et al., 2000; CDC, 2022a). In this study, serotyping was conducted to determine the serotypes of *Salmonella* that can be isolated from market hog lymph nodes and tonsils. These data are important assessing the risk these samples pose to human health.

4.2 Materials and methods

4.2.1 Sample collection

Of 2,437 market hog lymph nodes and tonsils that were tested for *Salmonella*, a total of 709 PCR- confirmed *Salmonella* isolates were recovered during a nationwide surveillance study conducted in 2022. Samples originated from three commerical pork processing facilties located around the U.S. All samples were collected from USDA-FSIS inspected and passed pork

carcasses. Sample collection occurred over three seasons (winter, spring and fall) in three regions of the U.S. (east, central, and west). Following *Salmonella* detection, isolates were frozen at Kansas State University for further characterization.

4.2.2 Sample processing

Isolates frozen at -80˚C in tryptic soy broth (TSB; BD Diagnostics, Franklin Lakes, NJ) with a 10% glycerol concentration were reactivated from frozen by streaking for isolation onto xylose lysine desoxycholate (XLD; Remel, Lenexa, KS) agar plates. The plates were incubated for 18-24 hours at 37˚C. Using a sterile needle, one colony characteristic of *Salmonella* (black and round) was transferred to a 1 mL tryptic soy agar (TSA; BD Diagnostics, Franklin Lakes, NJ) slant by stabbing the butt and streaking on the surface. All TSA slants were shipped to the United States Meat Animal Research Center (USMARC), a USDA Agricutlure Research Service research center in Clay Center, Nebraska, for *Salmonella* serotyping.

4.2.3 Serotyping

Serotypes were determined by using molecular serotyping methods (Echeita et al., 2002; Herrera-León et al., 2004; Herrera-León et al., 2007) and were confirmed by traditional slide agglutination (O typing) and tube agglutination (flagellar H typing) methods with commercial antisera (Difco, BD) following the manufacturer's guidelines.

4.3 Results and discussion

A total of 709 individual PCR *Salmonella*-positive isolates were serotyped, originating from 255 samples (three unique isolates were generated from each positive sample). From these

isolates, 30 different serotypes of *Salmonella* were identified. While a variety of serotypes that were recovered, the most frequently isolated serotype from these samples was monophasic *S.* Typhimurium (n=174 isolates), which accounted for nearly 25% of the isolates. This was followed by *S.* Agona (n=110 isolates) and *S.* Schwarzengrund (n=77 isolates), which represented 16% and 11% of the isolates. Table 4-1 lists each of these serotypes by number of isolates and samples. Other notable serotypes listed by frequency include *S.* Montevideo, Ohio, Infantis, Typhimurium, and Worthington.

As mentioned in Chapter 3, mesenteric LNs and tonsils had the highest prevalence of *Salmonella* among the seven sample types analyzed in this study. Figures 4-1 and 4-2 represent *Salmonella* serotypes isolated from mesenteric LNs and tonsils. A total of 290 of the 755 isolates originated from mesenteric LNs, with 73% of the Schwarzengrund isolates recovered from this sample type. Of the Agona and monophasic Typhimurium isolates, 34% and 32% were recovered from mesenteric LNs, respectively. A total of 254 of the 755 isolates originated from tonsils, representing 55% of the monophasic Typhimurium and 39% of the Agona isolates. Figure 4-3 represents isolates that were recovered from peripheral LNs (subiliac, superficial inguinal, pre-scapular, and axillary LNs). A total of 147 isolates originated from the peripheral LNs, where *S.* Montevideo was the most frequently isolated serotype (n=44). Peripheral LNs accounted for 96% of the *S.* Montevideo isolates in this study. *Salmonella* Agona, monophasic Typhimurium, and Ohio each had 24 isolates from peripheral LNs.

Table 4-1. *Salmonella* serotypes isolated, serotype frequency (by isolate and sample), and serotype prevalence (%) from market hog LNs and tonsils.

Serotype	Isolate Count	Sample Count	Prevalence (%) from Isolate Count
monophasic Typhimurium	174	61	24.5
Agona	110	39	15.5
Schwarzengrund	77	27	10.9
Montevideo	46	17	6.5
Ohio	46	16	6.5
Infantis	43	16	6.1
Typhimurium	38	14	5.4
Worthington	34	13	4.8
Give	27	9	3.8
Schleissheim	15	5	2.1
Muenchen	14	7	2.0
Cerro	12	4	1.7
Johannesburg	12	4	1.7
Braenderup	11	4	1.6
Mbandaka	8	3	1.1
London	4	2	0.6
Anatum	3	1	0.4
Berta	3	1	0.4
Djugu	3	1	0.4
Ealing	3	1	0.4
Hartford	3	1	0.4
monophasic Heidelberg	3	1	0.4
Muenster	3	1	0.4
Newport	3	1	0.4
Panama or Houston	3	1	0.4
Senftenberg	3	1	0.4
Thompson	3	1	0.4
Unknown monophasic, could be Mbandaka	3	1	0.4
Altona	1	1	0.1
Heidelberg	1	1	0.1
TOTAL	709	255	100.0

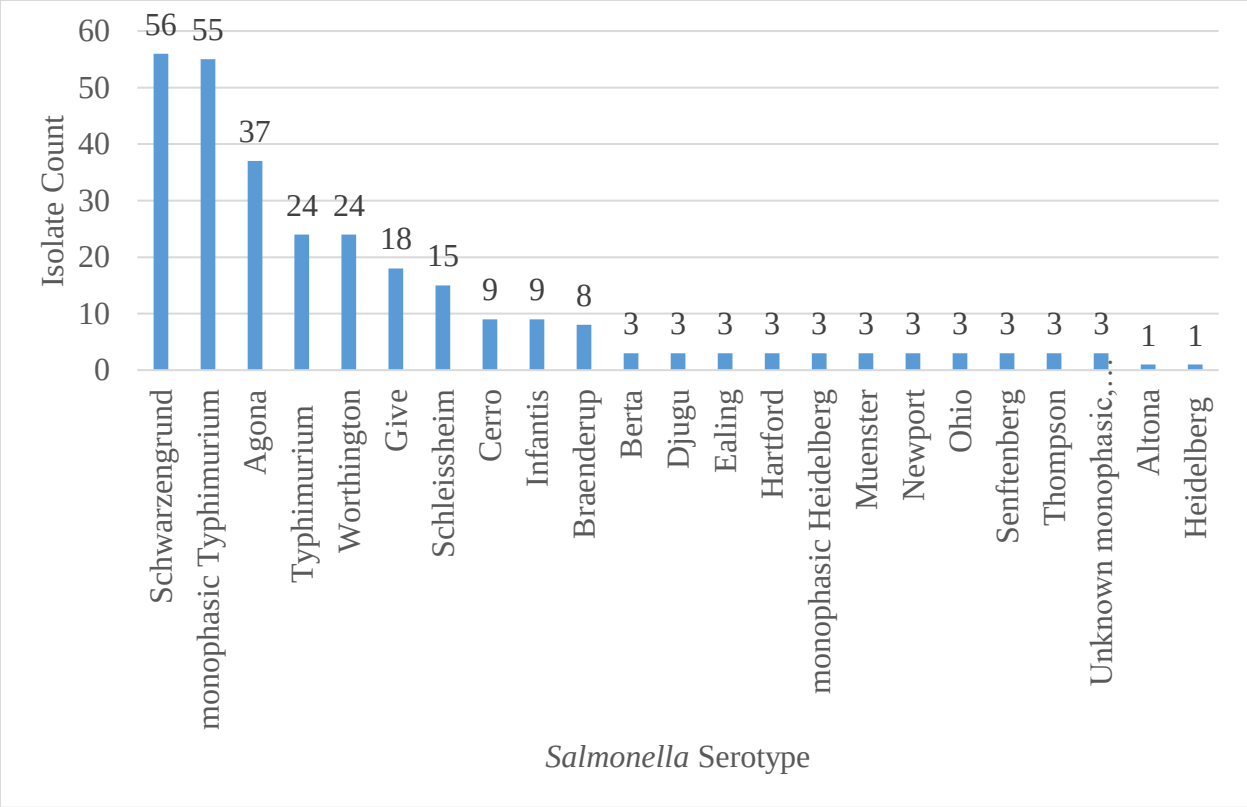

Figure 4-1. Graphical representation of *Salmonella* serotypes isolated from market hog mesenteric LNs organized by isolate count.

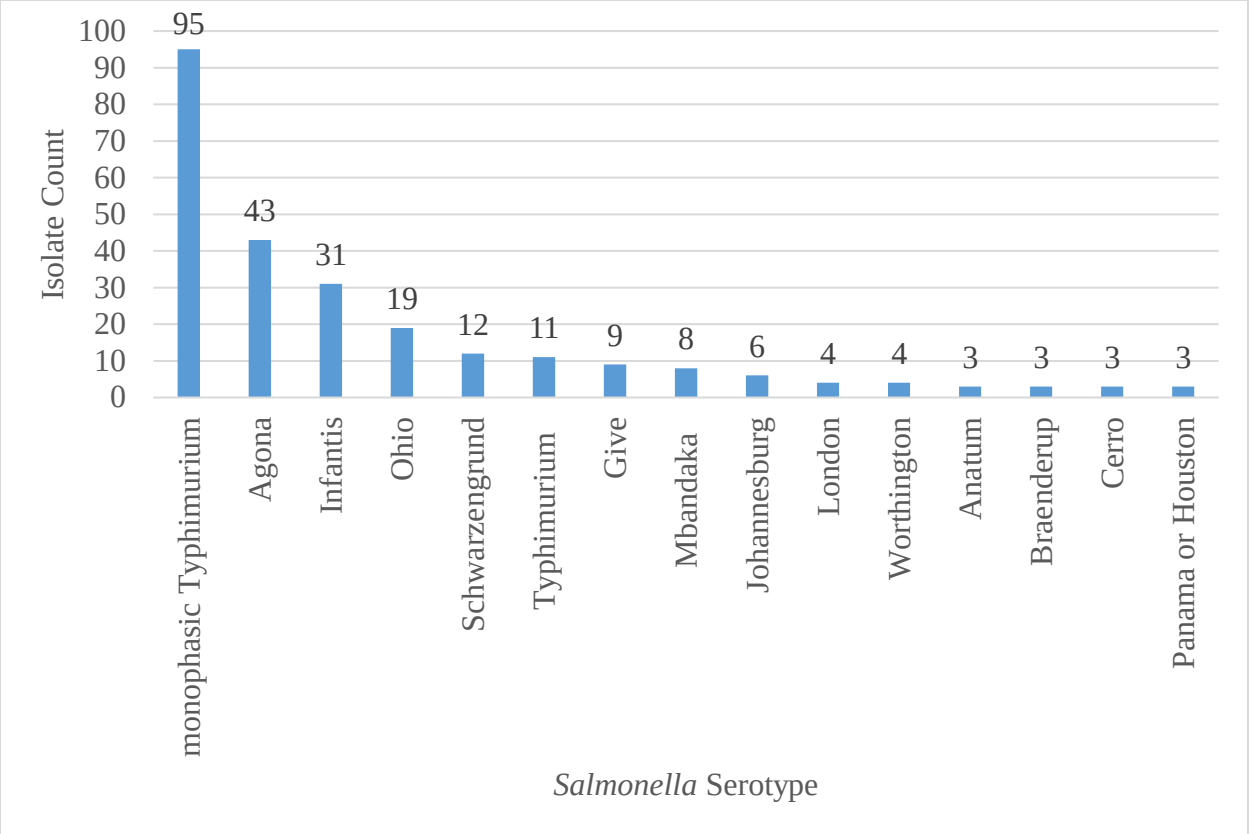

Figure 4-2. Graphical representation of *Salmonella* serotypes isolated from market hog tonsils organized by isolate count.

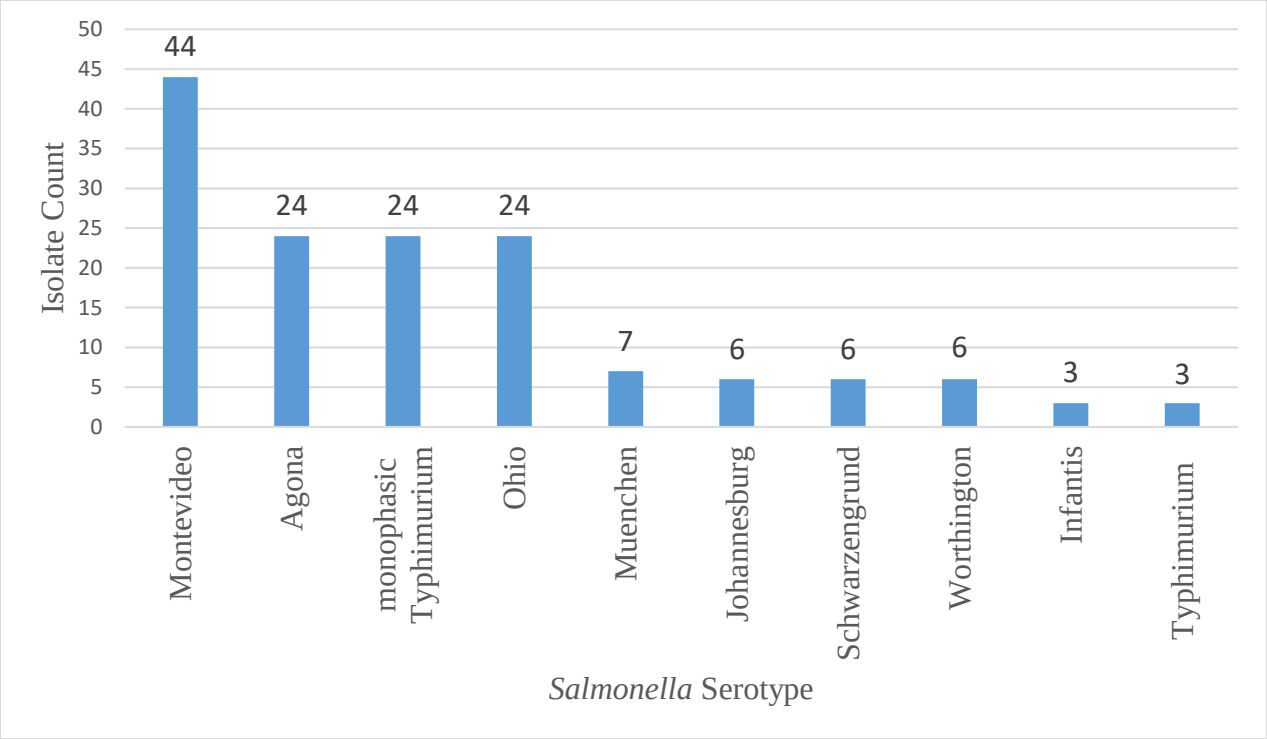

Figure 4-3. Graphical representation of *Salmonella* serotypes isolated from peripheral market hog LNs (subiliac, superficial inguinal, pre-scapular, and axillary LNs) organized by isolate count.

There are many serotypes presented in Table 4-1 that are pathogenic to humans. Monophasic *S.* Typhimurium is an ongoing challenge for the pork industry, both on farm and at the abattoir (Bonardi, 2017). Serotypes commonly isolated from pigs include Rissen, Panama, Brandenburg, Anatum, and Enteritidis (Bonardi, 2017). Frequently, *Salmonella* serotypes Typhimurium, monophasic Typhimurium, Derby, and Infantis have been isolated from hogs and pork meat products, all of which are serotypes that cause human salmonellosis, with some of these serotypes appearing in this study (Campos et al., 2019). Between 2014 and 2019, the USDA Food Safety Inspection Service reported more than 30 outbreaks of *Salmonella* from different serotypes related to a variety of pork products (USDA-FSIS, 2023).

Monophasic *S.* Typhimurium has appeared in several outbreaks related to pork products. It is a relatively new serotype that has been emerging in several locations in the world and is causing illness in both pigs and humans (Sun et al., 2020). Monophasic *S.* Typhimurium has been frequently associated with pigs, especially in the Europe and the United States (Bonardi, 2017; SHIC, 2021; Sun et al., 2020). Monophasic *S.* Typhimurium lacks the ability to express phase 2 of the flagellar H-antigen (Sun et al., 2020). This serotype can be multidrug resistant (MDR), raising concerns for global health; it is common for this strain to be resistant to ampicillin, streptomycin, sulfonamides, and tetracycline (SHIC, 2021). The full name for this strain is *Salmonella enterica* subsp. *enterica* 1,4,[5], 12:i:-. The formula can be understood as: 1 represents the subspecies; 4, 5, and 12 are O-antigens; i means that phase 1 H-antigen is present; - means phase 2 H-antigen is missing (SHIC, 2021).

In 2015, there was an outbreak of monophasic *S.* Typhimurium and *S.* Infantis in the northwestern section of the United States that resulted in 192 cases. Kapowsin Meats in Washington recalled more than 500,000 pounds of pork that may have been contaminated with these strains of *Salmonella*. The CDC National Antimicrobial Resistance Monitoring System (NARMS) conducted testing on 10 samples that were isolated from the clinical cases, and all were determined to be multidrug resistant. This is concerning from a global health perspective because infections from MDR strains may result in more hospitalizations and invasive infections (CDC, 2015). From 2018 to 2019, Denmark experienced an outbreak of monophasic *S.* Typhimurium that was linked to 49 cases. The products implicated included a raw Danish pork sausage, pork chops, and a ground pork and veal mixture; however statistical analysis estimated most infections came from the raw pork sausage that were likely undercooked (Helmuth et al., 2019). Another outbreak associated with monophasic *S.* Typhimurium occurred in France from

2020 to 2021 that resulted in 11 cases. Again, pork sausages were linked to the clinical cases. The three major serotypes that cause clinical cases in Europe are *S.* Enteritidis, Typhimurium, and monophasic Typhimurium. Pork products are estimated to cause about half of the salmonellosis cases each year in France (Pardos de la Gandara et al., 2023).

Salmonella Agona has been associated with clinical cases in the European Union (McCusker et al., 2014). This serotype has caused outbreaks in a variety of foods, including papayas, herbal tea, and rice and wheat puffs (CDC, 2008; Koch et al., 2005; Mba-Jonas et al., 2018). In 2014, there was an outbreak of *S.* Agona linked to 5 cases in California that were traced to pork. In 2015, another *S.* Agona pork-related outbreak was reported in Minnesota with 10 cases (USDA-FSIS, 2023). As shown in the outbreaks associated with this serotype, *S.* Agona can be a human pathogen, making it notable that *S.* Agona was isolated from these pork samples.

Harvey et al (2020) conducted a lymph node study from sows where *Salmonella* prevalence and serotyping was investigated. A total of 15 *Salmonella* serotypes were isolated, including Agona, Anatum, Enteritidis, Heidelberg, Johannesburg, Uganda, and Typhimurium. There were three notable serotypes: Derby, Brandenburg, and Infantis, which all exhibited multidrug resistance (Harvey et al., 2020). While drug resistance was not tested, *S.* Infantis was the fifth most isolated serotype in this present study (n=43). Bessire et al. (2018) conducted a similar study that also explored *Salmonella* serotype isolated from pork lymph nodes. Notable *Salmonella* serotypes from this study included London, Anatum, Worthington, Infantis, Johannesburg, Ohio and Uganda (Bessire et al., 2018). Several of these serotypes were again recovered in this present study, including *S.* London, Worthington, and Ohio. Moreover, a study was conducted investigating *Salmonella* in several types of lymph nodes and in feces. Four serotypes were discovered: Typhimurium, monophasic Typhimurium, Derby, and Brandenburg

(Guerra Filho et al., 2016). Monophasic *S.* Typhimurium and *S.* Typhimurium were isolated in this study as well. It is important to discuss the *Salmonella* serotypes that are isolated in various pork studies to show how often these serotypes appear and can be cause for concern from an epidemiological perspective.

Recently, a market hog lymph node study found *S.* Agona and Typhimurium in several phases as some of the most commonly isolated serotypes from lymph nodes (Larsen et al., 2023). It appears that monophasic *S.* Typhimurium is a problem that has been reported in several studies and has been present in the pork industry since the late 1990s (Harrison et al., 2022). Not only is a problem for hog producers and processing facilities, but it is a public health issue because it is disease inducing in humans. Most concerning about this serotype is its antimicrobial resistant properties (Harrison et al., 2022). *S.* Agona, along with several other serotypes, are also human pathogens that pose a risk to public health, especially for the strains that exhibit multidrug resistance. It appears that monophasic *S.* Typhimurium continues to present itself in pork systems, with this idea being reinforced by the number of isolates recovered in this study.

While there were a number of human pathogen serotypes that were recovered from tonsils and mesenteric LNs, these sample types are not usually associated with ground pork because they are removed from the carcass (from the head and viscera) during normal processing (Miller et al., 2022). Although, tonsils and mesenteric LNs may still be able to cause cross contamination to the carcass or knives during harvest. The serotypes found in the peripheral LNs (in Figure 4-3) are more relevant to the discussion of contamination from LNs in ground pork. Peripheral LNs are encased in fat in the carcass and remain untouched by harvest interventions (Gragg et al., 2013). Upon processing, these LNs can be exposed and combined with trim to make ground pork (Zhang et al., 2019). *Salmonella* Montevideo was the most frequently isolated

serotype from peripheral LNs, which is a human pathogen. While this serotype is commonly associated with poultry, there have been a variety of foodborne outbreaks that can be traced back to *S*. Montevideo (CDC, 2016, 2018; Lalsiamthara & Lee, 2017). While the majority of the isolates were recovered from tonsils and mesenteric LNs, the peripheral LNs still contributed to the pathogenic isolate counts. *Salmonella* contaminated peripheral LNs are a risk factor for ground pork, with these sample types harboring human pathogens in this study. From a public health standpoint, there should be concern for contamination from market hog LNs, specifically peripheral LNs because they can be found in ground pork (Miller et al., 2022; Vargas et al., 2023).

4.5 Conclusions

Salmonella serotypes that are pathogenic to humans can be isolated from market hog lymph nodes and tonsils. These sample types pose a risk to food safety due to cross contamination or direct contamination. Monophasic *S*. Typhimurium, *S.* Agona and *S*. Schwarzengrund were the most isolated serotypes of all sample types, all of which have been linked to several foodborne outbreaks. Monophasic *S*. Typhimurium appears to be a pathogen of concern especially in swine systems, from farm to table. From peripheral LNs that may contaminate ground pork, *S*. Montevideo was the most frequently isolated serotype. While the majority of the isolates were recovered from tonsils and mesenteric LNs, these sample types are less likely to contaminate ground pork because they are removed from the carcass. In terms of products that reach consumers, the isolates recovered from peripheral LNs are most concerning because they can be incorporated into ground pork. Serotyping of *Salmonella* allows for an understanding of emerging and present pathogens in the food industry to give insight into public

health. These data can be used by producers to conduct risk assessments and create mitigation strategies.

References

Acheson, D., & Hohmann, E. L. (2001). Nontyphoidal salmonellosis. *Clinical infectious diseases*, *32*(2), 263-269. https://doi.org/10.1086/318457

Bessire, B. C., Thomas, M., Gehring, K. B., Savell, J. W., Griffin, D. B., Taylor, T. M., Mikel, W. B., Campbell, J. A., Arnold, A. N., & Scaria, J. (2018). National survey of *Salmonella* prevalence in lymph nodes of sows and market hogs. *Translational animal science*, *2*(4), 365-371. https://doi.org/10.1093/tas/txy072

Bonardi, S. (2017). *Salmonella* in the pork production chain and its impact on human health in the European Union. *Epidemiology and infection*, *145*(8), 1513-1526. https://doi.org/10.1017/S095026881700036X

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan, B. (2000). *Salmonella* nomenclature. *Journal of Clinical Microbiology*, *38*(7), 2465-2467. https://doi.org/10.1128/jcm.38.7.2465-2467.2000

Campos, J., Mourão, J., Peixe, L., & Antunes, P. (2019). Non-typhoidal *Salmonella* in the pig production chain: a comprehensive analysis of its impact on human health. *Pathogens (Basel)*, *8*(1), 19. https://doi.org/10.3390/pathogens8010019

Canada, P. H. A. o. (2010). *Pathogen safety data sheets: infectious substances – Salmonella enterica spp.* Government of Canada. https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/salmonella-enterica.html#note4

CDC. (2008, 13 May 2008). *Multistate outbreak of Salmonella Agona infections linked to rice and wheat puff cereal (final update)*. Centers for Disease Control and Prevention. https://www.cdc.gov/salmonella/2008/rice-wheat-puff-cereal-5-13-2008.html

CDC. (2015, December 2, 2015). *Multidrug-resistant Salmonella I 4,[5],12:i:- and Salmonella Infantis infections linked to pork, 2015*. CDC. https://www.cdc.gov/salmonella/pork-08-15/index.html

CDC. (2016, 20 May 2016). *Salmonella Montevideo and Salmonella Senftenberg infections linked to wonderful pistachios, 2016*. https://www.cdc.gov/salmonella/montevideo-03-16/index.html

CDC. (2018, 28 February 2018). *Salmonella Montevideo infections linked to raw sprouts, 2018*. https://www.cdc.gov/salmonella/montevideo-01-18/index.html

CDC. (2022a, September 9, 2022). *Serotypes and the importance of serotyping*. CDC. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html

CDC. (2022b, 08 July 2022). *Snapshots of Salmonella serotypes*. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotype-snapshots.html

CDC. (2023, 23 May 2023). *Salmonella*. Centers for Disease Control and Prevention. https://www.cdc.gov/salmonella/index.html

Echeita, M. A., Herrera, S., Garaizar, J., & Usera, M. A. (2002). Multiplex PCR-based detection and identification of the most common *Salmonella* second-phase flagellar antigens.

Research in Microbiology, *153*(2), 107-113. https://doi.org/10.1016/s0923-2508(01)01295-5

Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G., & Lee, L.-H. (2015). *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in life science*, *8*(3), 284-293. https://doi.org/10.1080/21553769.2015.1051243

Ferrari, R. G., Rosario, D. K. A., Cunha-Neto, A., Mano, S. B., Figueiredo, E. E. S., & Conte-Junior, C. A. (2019). Worldwide epidemiology of *Salmonella* serovars in animal-based foods: a meta-analysis. *Applied and Environmental Microbiology*, *85*(14). https://doi.org/10.1128/AEM.00591-19

Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella* enterica serovars differ. *Frontiers in microbiology*, *5*, 391-391. https://doi.org/10.3389/fmicb.2014.00391

Giannella, R. A. (1996). *Medical microbiology* (S. Baron, Ed. 4th ed.) https://www.ncbi.nlm.nih.gov/books/NBK8435/

Gong, B., Li, H., Feng, Y., Zeng, S., Zhuo, Z., Luo, J., Chen, X., & Li, X. (2022). Prevalence, serotype distribution and antimicrobial resistance of non-typhoidal *Salmonella* in hospitalized patients in Conghua District of Guangzhou, China. *Frontiers in cellular and infection microbiology*, *12*, 805384-805384. https://doi.org/10.3389/fcimb.2022.805384

Gragg, S. E., Loneragan, G. H., Brashears, M. M., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Wang, R., Schmidt, J. W., Brooks, J. C., Shackelford, S. D., Wheeler, T. L., Brown, T. R., Edrington, T. S., & Brichta-Harhay, D. M. (2013). Cross-sectional

study examining *Salmonella* enterica carriage in subiliac lymph nodes of cull and feedlot cattle at harvest. *Foodborne pathogens and disease*, *10*(4), 368-374. https://doi.org/10.1089/fpd.2012.1275

Guerra Filho, J. B. P., Yamatogi, R. S., Possebon, F. S., Fernandes, S. A., Tiba-Casas, M. R., Lara, G. H. B., Ribeiro, M. G., & Pinto, J. P. A. N. (2016). Frequency, serotyping and antimicrobial resistance pattern of *Salmonella* from feces and lymph nodes of pigs. *Pesquisa Veterinária Brasileira*, *36*(12), 1165-1170. https://doi.org/10.1590/s0100-736x2016001200004

Gut, A. M., Vasiljevic, T., Yeager, T., & Donkor, O. N. (2018). *Salmonella* infection - prevention and treatment by antibiotics and probiotic yeasts: a review. *Microbiology (Society for General Microbiology)*, *164*(11), 1327-1344. https://doi.org/10.1099/mic.0.000709

Harrison, O. L., Gebhardt, J. T., Paulk, C. B., Plattner, B. L., Woodworth, J. C., Rensing, S., Jones, C. K., & Trinetta, V. (2022). Inoculation of weaned pigs by feed, water, and airborne transmission of *Salmonella* enterica serotype 4,[5],12:i. *Journal of food protection*, *85*(4), 693-700. https://doi.org/10.4315/JFP-21-418

Harvey, R. B., Norman, K. N., Anderson, R. C., & Nisbet, D. J. (2020). A preliminary study on the presence of *Salmonella* in lymph nodes of sows at processing plants in the United States. *Microorganisms (Basel)*, *8*(10), 1602. https://doi.org/10.3390/microorganisms8101602

Helmuth, I. G., Espenhain, L., Ethelberg, S., Jensen, T., Kjeldgaard, J., Litrup, E., Schjørring, S., & Müller, L. (2019). An outbreak of monophasic *Salmonella* Typhimurium associated with raw pork sausage and other pork products, Denmark 2018-19. *Epidemiology and infection*, *147*, e315-e315. https://doi.org/10.1017/S0950268819002073

Herrera-León, S., McQuiston, J. R., Usera, M. A., Fields, P. I., Garaizar, J., & Echeita, M. A. (2004). Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *Journal of Clinical Microbiology*, *42*(6), 2581-2586. https://doi.org/10.1128/jcm.42.6.2581-2586.2004

Herrera-León, S., Ramiro, R., Arroyo, M., Díez, R., Usera, M. A., & Echeita, M. A. (2007). Blind comparison of traditional serotyping with three multiplex PCRs for the identification of *Salmonella* serotypes. *Research in Microbiology*, *158*(2), 122-127. https://doi.org/10.1016/j.resmic.2006.09.009

Hurd, H. S., McKean, J. D., Wesley, I. V., & Karriker, L. A. (2001). The Effect of lairage on *Salmonella* isolation from market swine. *Journal of food protection*, *64*(7), 939-944. https://doi.org/10.4315/0362-028X-64.7.939

Koch, J., Schrauder, A., Alpers, K., Werber, D., Frank, C., Prager, R., Rabsch, W., Broll, S., Feil, F., Roggentin, P., Bockemühl, J., Tschäpe, H., Ammon, A., & Stark, K. (2005). *Salmonella* agona outbreak from contaminated aniseed, Germany. *Emerging infectious diseases*, *11*(7), 1124-1127. https://doi.org/10.3201/eid1107.041022

Lalsiamthara, J., & Lee, J. H. (2017). Pathogenic traits of *Salmonella* Montevideo in experimental infections in vivo and in vitro. *Scientific reports*, *7*(1), 46232-46212. https://doi.org/10.1038/srep46232

Larsen, S., Bokenkroger, C., Delago, J., & Radtke, D. (2023, 15 May 2023). *Salmonella enterica prevalence and serotype distribution in lymph nodes from market swine* SafePork 2023, New Orleans, LA. https://www.iastatedigitalpress.com/safepork/article/id/16359/

Matthews, K. R., Kniel, K. E., & Montville, T. J. (2017). *Food microbiology- an introduction (4th Edition)* (4th ed.). American Society for Microbiology (ASM).

Mba-Jonas, A., Culpepper, W., Hill, T., Cantu, V., Loera, J., Borders, J., Saathoff-Huber, L., Nsubuga, J., Zambrana, I., Dalton, S., Williams, I., & Neil, K. P. (2018). A multistate outbreak of human *Salmonella* Agona infections associated with consumption of fresh, whole papayas imported from Mexico-United States, 2011. *Clinical infectious diseases*, *66*(11), 1756-1761. https://doi.org/10.1093/cid/cix1094

McCusker, M. P., Hokamp, K., Buckley, J. F., Wall, P. G., Martins, M., & Fanning, S. (2014). Complete genome sequence of *Salmonella* enterica serovar Agona pulsed-field type SAGOXB.0066, cause of a 2008 pan-European outbreak. *Genome announcements (Washington, DC)*, *2*(1). https://doi.org/10.1128/genomeA.01219-13

McQuiston, J. R., Waters, R. J., Dinsmore, B. A., Mikoleit, M. L., & Fields, P. I. (2011). Molecular determination of H antigens of *Salmonella* by use of a microsphere-based liquid array. *Journal of Clinical Microbiology*, *49*(2), 565-573. https://doi.org/10.1128/JCM.01323-10

Miller, M., Maher, J. M., Wiseman, B., & Gragg, S. E. (2022). *Salmonella* is present in multiple lymph nodes of market hog carcasses at slaughter. *Food protection trends*, *42*(2), 100-106. https://doi.org/https://doi.org/10.4315/FPT-21-027

Pardos de la Gandara, M., Fournet, N., Bonifait, L., Lefèvre, S., Chemaly, M., Grastilleur, C., Cadel-Six, S., Fach, P., Pignault, A., Brisabois, A., Jourdan-Da Silva, N., & Weill, F.-X. (2023). Countrywide multi-serotype outbreak of *Salmonella* Bovismorbificans ST142 and monophasic *Salmonella* Typhimurium ST34 associated with dried pork sausages in France, September 2020 to January 2021. *Euro Surveillance: Bulletin Européen sur les Maladies Transmissibles*, *28*(2), 1. https://doi.org/10.2807/1560-7917.ES.2023.28.2.2200123

SHIC. (2021, April 2021). *Salmonella enterica subsp. enterica 1,4,[5],12:i:-*. Swine Health Information Center. https://www.swinehealth.org/wp-content/uploads/2021/04/Salmonella-14512i-.pdf

Sun, H., Wan, Y., Du, P., & Bai, L. (2020). The epidemiology of monophasic *Salmonella* Typhimurium. *Foodborne pathogens and disease*, *17*(2), 87-97. https://doi.org/10.1089/fpd.2019.2676

Teunis, P. F. M., Kasuga, F., Fazil, A., Ogden, I. D., Rotariu, O., & Strachan, N. J. C. (2010). Dose–response modeling of *Salmonella* using outbreak data. *International journal of food microbiology*, *144*(2), 243-249. https://doi.org/10.1016/j.ijfoodmicro.2010.09.026

USDA-FSIS. (2023, 01 June 2023). *FSIS guideline to control Salmonella in swine slaughter and pork processing establishments* US Department of Agriculture Food Safety and

Inspection Service. https://www.fsis.usda.gov/sites/default/files/media_file/documents/FSIS-GD-2023-0003.pdf

Vargas, D. A., Betancourt-Barszcz, G. K., Blandon, S. E., Applegate, S. F., Brashears, M. M., Miller, M. F., Gragg, S. E., & Sanchez-Plata, M. X. (2023). Rapid quantitative method development for beef and pork lymph nodes using BAX ® system real time *Salmonella* Assay. *Foods*, *12*(4).

Wain, J., House, D., Zafar, A., Baker, S., Nair, S., Kidgell, C., Bhutta, Z., Dougan, G., & Hasan, R. (2005). Vi antigen expression in *Salmonella* enterica serovar Typhi clinical isolates from Pakistan. *Journal of Clinical Microbiology*, *43*(3), 1158-1165. https://doi.org/10.1128/JCM.43.3.1158-1165.2005

WHO. (2022, May 19, 2022). *Food safety*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/food-safety#:~:text=Salmonella%2C%20Campylobacter%20and%20enterohaemorrhagic%20Escherichia,with%20severe%20and%20fatal%20outcomes.

Zhang, Y., O'Connor, A. M., Wang, C., Dickson, J. S., Hurd, H. S., & Wang, B. (2019). Interventions targeting deep tissue lymph nodes may not effectively reduce the risk of salmonellosis from ground pork consumption: a quantitative microbial risk assessment. *Risk analysis*, *39*(10), 2237-2258. https://doi.org/10.1111/risa.13317

Chapter 5 - Summary and Conclusions

5.1 *Salmonella* in market hog lymph nodes

Research has shown that pathogenic *Salmonella* is present in several types of market hog lymphatic tissue. *Salmonella* was detected in all seven sample types analyzed in the first study. In general, *Salmonella* prevalence was highest in the eastern region and during the spring months, though statistical significance varied based on sample type. Quantification results were only statistically analyzed for mesenteric LNs and tonsils. The average *Salmonella* concentration in mesenteric LNs ranged from 0.7 $\log_{10}$ CFU/sample (western region in the fall) to 3.1 $\log_{10}$ CFU/sample (western region in the winter), and concentration varied by region and season (P=0.014). The average *Salmonella* concentration in tonsils ranged from 1.1 $\log_{10}$ CFU/sample (central region in the fall) to 3.0 $\log_{10}$ CFU/sample (western region in the winter), and concentration varied by region and season (P=0.031).

The 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant® enumeration methods were compared for their ability to enumerate market hog lymph nodes and tonsils. Both methodologies were used for all sample types (excluding tonsils) to determine the differences between enumeration values. The kappa value (measurement for agreement) for the results was 0.384 and is statistically significant (P<0.001), which indicates that both methods can be used to quantify *Salmonella*.

Isolates recovered from market hog lymph nodes and tonsils were serotyped, with *S.* Agona and monophasic *S.* Typhimurium accounting for most isolates, at 25% and 21%, respectively. Both are human pathogens, and monophasic *S.* Typhimurium is of particular concern for pork producers and processors. Several monophasic *S.* Typhimurium outbreaks have been linked to

pork products across the globe. Other serotypes associated with outbreaks of human illness were recovered, further reaffirming that lymphoid tissues pose a risk to food safety.

Considering the process of a *Salmonella* infection, it is not unexpected that mesenteric LNs have the second highest prevalence and highest enumeration value. During host invasion by *Salmonella*, cells can multiply and spread to lymph nodes, most notably the mesenteric LN due to their location in the viscera (i.e., proximity to the gut). Briefly, after *Salmonella* has attached to epithelial cells of the host, dendritic cells move *Salmonella* from the lymph fluid into LNs located in the gastrointestinal tract. The ability of *Salmonella* to establish and survive in lymphatic tissue is unique; the lymph system is responsible for filtering bacterial and viral pathogens from the lymph fluid that circulates in the body. Lymph nodes are part of the immune system, and the role is to filter and subdue bacteria and viruses found in the body (Arthur et al., 2008; Vargas et al., 2023). Due to the curious nature of hogs, they tend to chew on many things in their environment (i.e., lairage ropes), resulting in ingestion by fecal-oral route. Tonsils are located near the mouth of the hog, which may explain why this lymph tissue had the highest overall *Salmonella* prevalence in this study.

5.2 Suggested further research

Pork is reported to be the most consumed type of meat in the world and has caused a multitude of foodborne outbreaks. *Salmonella* presence and contamination can be reduced following on-farm strategies and proper slaughter techniques (Baer et al., 2013). Further research should be conducted to establish the risk of lymphoid tissues causing human salmonellosis. There are gaps in knowledge for *Salmonella* prevalence in spleens and livers, though these are important lymphoid tissues. However, these are not widely consumed and can be considered

offal by-products in the pork industry. A deeper dive into the process of *Salmonella* spread amongst peripheral lymph nodes, as well as quantification of this pathogen, may prove beneficial for risk analysis. Moreover, tracking serotypes from swine houses through slaughter may prove beneficial for understanding contamination processes from farm to fork. The effect of lairage on introducing pathogens such as *Salmonella* to clean hogs is also a concern. Effective methods to reduce pathogen loads during transportation and in holding pens should be developed (USDA-FSIS, 2023).

Identification of the most effective measures to reduce contamination from market hog lymph nodes and other lymph tissues is ongoing. However, several sources report that removal of LNs from the carcass may be the best course of action. There are issues with this suggested approach; removal will require a lot of time and skill to prevent contamination during this process, not to mention that line speed could interfere with proper techniques (Vargas et al., 2023). Methods that exclude LNs from product destined for comminuted pork may be effective *Salmonella* mitigation strategies (USDA-FSIS, 2023).

During the quantification method comparison between 3M™ EB Petrifilm™ + XLD replica plate method and BAX®-System-SalQuant®, it was mentioned that a new curve should be developed to manage tonsil quantification with BAX®-System-SalQuant®. Tonsils enumerated in Chapter 3 utilized the lymph node quantification curve for BAX®-System-SalQuant®. Following plating on 3M™ EB Petrifilm™ + XLD replica plate method, it was noted that tonsils were highly contaminated, and the background flora interfered with proper enumeration. A separate tonsil quantification curve should be developed with BAX®-System-SalQuant® and quant solution should be used to reduce interference from background flora.

References

Arthur, T. M., Brichta-Harhay, D. M., Bosilevac, J. M., Guerini, M. N., Kalchayanand, N., Wells, J. E., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2008). Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. *Journal of food protection*, *71*(8), 1685-1688. https://doi.org/10.4315/0362-028X-71.8.1685

Baer, A. A., Miller, M. J., & Dilger, A. C. (2013). Pathogens of interest to the pork industry: a review of research on interventions to assure food safety. *Comprehensive reviews in food science and food safety*, *12*(2), 183-217. https://doi.org/10.1111/1541-4337.12001

USDA-FSIS. (2023, 01 June 2023). *FSIS guideline to control Salmonella in swine slaughter and pork processing establishments* US Department of Agriculture Food Safety and Inspection Service. https://www.fsis.usda.gov/sites/default/files/media_file/documents/FSIS-GD-2023-0003.pdf

Vargas, D. A., Betancourt-Barszcz, G. K., Blandon, S. E., Applegate, S. F., Brashears, M. M., Miller, M. F., Gragg, S. E., & Sanchez-Plata, M. X. (2023). Rapid quantitative method development for beef and pork lymph nodes using BAX ® system real time *Salmonella* Assay. *Foods*, *12*(4).