Salmonella quantification (SalQuant®) utilizing BAX® system for pork primary production samples and exploring the use of Probicon L28 and BIOPLUS® 2B as direct-fed microbials to reduce *Salmonella* and Shiga toxin-producing *Escherichia coli* in market pigs

by

Jimeng Bai

B.S., Kansas State University, 2021

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

Major Professor
Sara E. Gragg, Ph.D.

Copyright

© Jimeng Bai 2023.

Abstract

Salmonella is a common pathogen in pigs, and pork has been associated with causing salmonellosis in humans. Because pigs infected with pathogens can transport the pathogens to the abattoir and contaminate pork products, the identification and validation of a rapid and effective method for the detection and quantification of *Salmonella* in pre-harvest and harvest samples would benefit the pork industry. Because *Salmonella* is commonly found in the intestines and lymph nodes of pigs, as well as farm environments, it is possible to quantify *Salmonella* using pre-harvest and harvest samples. The first study validated SalQuant® System as a polymerase chain reaction (PCR) assay for the quantification of *Salmonella* in boot covers, ropes, spleens, and pig feces. Samples negative for *Salmonella* were inoculated with *Salmonella* Typhimurium 14028 strains at 0 to 4 or 0 to 8 log CFU/g and samples were subjected to the BAX® Real-Time *Salmonella* assay after 0 (5 to 8 log CFU/g samples only), 6, 8, and 10 hours of incubation at 42°C. Three biological replicates and 5 technical replicates were completed for each sample type, and one biological replicate was also enumerated using the most probable number (MPN) technique as a comparison. After data collection, a linear fit equation was established for all incubation times, with $R^2 > 0.80$, log RMSE < 0.60 used as the threshold for identifying the incubation time that is most effective for quantifying *Salmonella*. A paired t-test compared the *Salmonella* concentrations generated by MPN and SalQuant® for one biological replication, and the two methods were not significantly different ($P>0.05$) for all sample types except spleens ($P=0.0246$).

Shiga toxin-producing *Escherichia coli* (STEC) is another pathogen that can be found in pigs and in their farm environments. Identifying effective pre-harvest interventions can reduce pathogens in market pigs to reduce the burden of pathogens entering the abattoir. Many studies

have demonstrated that *Lactobacillus* is an effective pre-harvest intervention in cattle, particularly in controlling *Escherichia coli* O157:H7. *Lactobacillus salivarius* L28 (Probicon L28) is a patented probiotic that can be given to animals as a pre-harvest food safety intervention to reduce pathogens. BIOPLUS® 2B is a probiotic comprised of *Bacillus subtilis* and *Bacillus licheniformis*. It is commonly used in the pig industry to establish gastrointestinal microbiota and promote animal health. The purpose of the second study was to investigate BIOPLUS® 2B and Probicon L28 as pre-harvest interventions to reduce *Salmonella* and Shiga toxin-producing *E. coli* in market pigs. Two groups of market pigs (N=294 pigs in group 1; N=356 in group 2, initial weight = 116.2 lb) were enrolled in the study. A total of 36 pens were used for each group of pigs and pigs were assigned at random to pen. Each pen was assigned to one of three treatments, with a total of 12 pens per treatment: a control treatment consisting of a standard corn-soybean meal (SBM) finishing diet (no probiotic), a standard corn-SBM finishing diet supplemented with Probicon L28 (target concentration of 1.0×10^6 CFU/head/day) through water lines using a water medication system, a standard corn-SBM finishing diet supplemented with BIOPLUS® 2B at a target concentration of 3.0×10^9 CFU/head/day. Each group of pigs was sampled at three stages during the feeding period (arrival/baseline, halfway/6 weeks and prior to loadout/13 weeks), and at each stage the following samples were collected: feces collected from four pigs at random in each pen, two boot covers per pen, and one rope sample per pen. Market pigs were followed to the abattoir and superficial inguinal lymph nodes were collected from each pig. The BAX® System was used to detect *Salmonella, E. coli* O157:H7, and Shiga toxin-producing *E. coli* (*stx*, *eae* genes, serogroups O26, O121, O45, O103 and O145). In this study, *Salmonella* prevalence was very low at less than 3% for all sample types collected from group 1 and 2 pigs. Dietary supplementation of BIOPLUS® 2B and Probicon L28 in finishing pigs had no effect (P>0.05) on

the prevalence of STEC (*stx* and *eae* genes) or serogroups O26, O121, O45, O103 and O145 in pig feces, boot covers, and ropes prior to loadout, or lymph nodes collected at the abattoir.

Table of Contents

List of Figures ... viii

List of Tables ... ix

Acknowledgements ... x

Chapter 1 - Introduction and Research Questions ... 1

References: ... *4*

Chapter 2 - Literature Review ... 8

2.1 Salmonella background information ... *8*

2.2 Importance of Salmonella Research ... *10*

Salmonella *in Swine* ... 10

Salmonella *in Pork* ... 12

Salmonella *Outbreaks in Pork* ... 14

Economic Impact of Salmonella ... 15

2.3 Shiga toxin-producing Escherichia coli (STEC) background information ... *15*

2.4 Importance of STEC Research ... *18*

STEC in Swine ... 18

STEC in Pork ... 19

STEC Outbreaks in Pork ... 20

Public Health Impact of STEC ... 21

2.5 Pre-harvest Interventions ... *21*

Probiotics ... 21

Probicon L28 （Lactobacillus salivarius L28） ... 23

BIOPLUS® 2B ... 24

2.6 Pathogen Control Actions ... *24*

2.7 Enumeration Methods ... *25*

Directing Plating ... 25

3M™ Petrifilm™ ... 26

Most Probable Number ... 27

Real-Time Polymerase Chain Reaction ... 28

BAX® System SalQuant® ... 29

References: ... *30*

Chapter 3 - *Salmonella* Quantification Utilizing SalQuant® and the BAX® System for Swine and Pork Samples ... 49

Abstract ... *49*

3.1 Introduction ... *50*

3.2 Materials and Methods ... *52*

3.2.1 Experimental Design ... 52

3.2.2 Sample Collection ... 52

3.2.3 Inoculation Procedures ... 53

3.2.4 Sample Processing and Curve Development ... 54

3.2.5 Curve Verification ... 56

3.2.6 Statistical Analysis ... 57

3.3 Results ... ***57***

3.3.1 Curve Development ... 57

3.3.2 Curve Verification ... 58

3.4 Discussion ... ***59***

3.5 Conclusions ... ***61***

3.6 Figures and Tables ... ***61***

References ... ***67***

Chapter 4 - Exploring the Use of Probicon L28 and BIOPLUS® 2B as Direct-Fed Microbials to Reduce *Salmonella* and Shiga Toxin-Producing *Escherichia coli* in Market Pigs ... **70**

Abstract ... ***70***

4.1 Introduction ... ***71***

4.2 Materials and methods ... ***73***

4.2.1 Experimental Design ... 73

4.2.2 Treatment Diets ... 75

4.2.3 Pre-Harvest Sample Collection ... 76

4.2.4 Harvest Sample Collection ... 77

4.2.5 Pre-Harvest Sample Processing ... 78

4.2.6 Harvest Sample Collection ... 78

4.2.7 STEC and Salmonella *Detection* ... 79

4.2.8 STEC and Salmonella *Isolation and Characterization* ... 80

4.2.9 STEC and Salmonella *Serotyping* ... 81

4.2.10 Statistical Analysis ... 82

4.3 Results ... ***83***

4.3.1 STEC and Serogroup Prevalence ... 83

4.3.2 Salmonella *Prevalence* ... 85

4.3.3 STEC and Salmonella *Isolation* ... 86

4.4 Discussion ... ***87***

4.5 Conclusion ... ***90***

4.6 Figures and tables ... ***91***

References: ... ***98***

Chapter 5 - Conclusion ... **103**

References: ... ***104***

List of Figures

Figure 3-1. SalQuant® Curve Development Experimental Design for Pig Feces (Low and High Level), Spleen, Rope, and Boot Cover Samples 61
Figure 3-2. SalQuant® Curve Estimations of *Salmonella* Typhimurium 14028 in Log_{10} CFU/mL of Boot Covers Collected from a Market Pig Barn According to Inoculation Concentration (Log_{10} CFU/mL). 62
Figure 3-3. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Spleens Collected from a Large Commercial USDA-FSIS Inspected Pork Producer According to Inoculation Concentration (Log_{10} CFU/mL). 62
Figure 3-4. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Ropes Collected from a Large Commercial USDA-FSIS Inspected Pork Producer According to Inoculation Concentration (Log_{10} CFU/mL). 63
Figure 3-5 SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Pig Feces Collected from a Market Pig Barn by Inoculation Level 0-4 Log_{10} CFU/mL 63
Figure 3-6. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Pig Feces Collected from a Market Pig Barn by Inoculation Level 5-8 Log_{10} CFU/mL 64
Figure 4-1. Barn Map of Finishing Diets Assigned to Pens of Group 1 Market Pigs (8-10 pigs per pen) During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet. 91
Figure 4-2. Barn Map of Finishing Diets Assigned to Pens of Group 2 Market Pigs (8-10 pigs per pen) During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet. 92

List of Tables

Table 3-1. BAX® System SalQuant® Curve Development of *Salmonella* Typhimurium 10428 Parameters of Boot Cover, Ropes, Spleen, and Pig feces Matrices 65
Table 3-2. BAX® System SalQuant® Linear-Fit Curve Estimations of *Salmonella* Typhimurium 10428 Compared to MPN Estimations for Matched Biological Replication Samples. . . . 66
Table 4-1. Treatment Effects in the Analyses of Shiga toxin-producing *Escherichia coli* (STEC), *E. coli* O26 and *E. coli* O121 Prevalence from the Feces of Pigs Fed Control, BIOPLUS® 2B and Probicon Diets for 6 and 13 Weeks During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.. 93
Table 4-2.Treatment Effects in the Analyses of STEC Prevalence Status for LN Samples During Harvest Sample Collection. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet. 94
Table 4-3. STEC Serotypes Isolated from Lymph nodes and Trim from Group 1 and Group 2. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.. 95
Table 4-4. *Salmonella* Serotypes Isolated from Pig Feces, Boot Covers, Ropes, Lymph nodes, MicroTally™, and Trim from Group 1. Baseline: Upon Arrival to the Finishing Barn. 6 weeks: Approximately Midway on Trial. 13 weeks: Before Loadout. 96
Table 4-5. *Salmonella* Serotypes Isolated from Pig Feces, Boot Covers, Ropes, Lymph nodes, MicroTally™, and Trim from Group 2. Baseline: Upon Arrival to the Finishing Barn. 6 weeks: Approximately Midway on Trial. 13 weeks: Before Loadout. 97

Acknowledgements

I would like to express my sincere thanks to my advisors, Dr. Sara Gragg, for giving me the wonderful opportunity to work with her and pursue my degree at Kansas State University. I also want to thank my committee members Dr. Jessie Vipham, Dr. Jordan Gebhardt, and Dr. Savannah Applegate for guidance and assistance in the studies. Thanks to Dr. Qing Kang for her help with statistical analysis of my feeding trial study. Thanks to Dr. John Schmidt and Dr. Joseph (Mick Bosilevac) from USMARC for isolate characterization and other contributions to my research.

I am very grateful to my team of graduate, undergraduate and internship students for their help and friendship during my work. The support, friendship, and encouragement I have received from this team have been invaluable to me.

I sincerely thank Macie Reeb and Swine Teaching & Research Center at Kansas State University for assistance with all aspects of this study.

I also want to thank the Foundation for Meat & Poultry Research & Education, National Pork Checkoff, Safe Foods, and Hygiena for providing funding for my research. I also thank NexGen Probiotics, LLC for providing the Probicon L28 product for my feeding trial.

Most importantly, I want to thank my family, especially my parents, Gang Bai and Qiuhui Ge. They encouraged me to pursue my dreams and supported me to complete my studies.

Chapter 1 - Introduction and Research Questions

Salmonella, Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria monocytogenes* are believed to be the leading causes of foodborne illness outbreaks in the United States (Marshall et al., 2020). Shiga toxin-producing *E. coli* and *Salmonella* are associated with the most hospitalizations or deaths (Scott et al., 2020). Cattle, chickens, pigs and turkeys are major reservoirs of *Salmonella* and STEC, both of which are considered zoonotic diseases that can cause illness and death in humans, which is why these pathogens are a serious concern for public health (Heredia & García, 2018). According to the U.S. Centers for Disease Control and Prevention (CDC), there are an estimated 525,000 foodborne infections, 2,900 hospitalizations, and 82 deaths from eating pork each year (Fernandez et al., 2021; Self et al., 2017), which suggests that efforts to rapidly detect, quantify, and reduce foodborne pathogens in pork products is of high Importance.

Salmonella enterica is a bacterium of importance to pig farmers because *Salmonella* infections can cause serious illness in pigs and contaminated pork products can affect human health (Schwartz, 2021). Of the 2,500 serotypes of *Salmonella*, only a few cause illness in pigs, and all of these serotypes have the potential to cause food poisoning in humans (Schwartz, 2021). When salmonellosis occurs in pigs, it is usually associated with *Salmonella* Typhimurium, *Salmonella* Heidelberg, and *Salmonella* Choleraesuis (Schwartz, 2021). Most people infected with *Salmonella* develop diarrhea, fever and stomach cramps, but some patients require hospitalization (Centers for Disease Control and Prevention, 2023).

There is an urgent need to develop and validate a rapid and effective method for the detection and quantification of *Salmonella* in the pork industry. The most probable number (MPN) technique can be used to quantitatively estimate bacteria in food or water samples

(Chandrapati & Williams, 2014). The traditional enumerative MPN method is a statistical method based on the principle of dilution to extinction (diluted by a factor of 10), and the sample dilutions are used to inoculate a a broth to estimate the number of live bacteria in the samples. (Karunasagar et al., 2018). The MPN technique has many disadvantages because it requires a lot of glassware and materials, is labor-intensive and time-consuming, and results lack sensitivity (Erkmen, 2022). Quantitative, real-time polymerase chain reaction (qPCR) is a rapid and efficient method to detect and quantify target DNA sequences in different substrates (Kralik & Ricchi, 2017). Real-Time PCR has several advantages, including improved sensitivity and the ability to 1) process multiple samples at same time, and 2) provide immediate information (Hoy, 2013).

Escherichia coli resides in the intestines of humans and animals, and most strains are harmless; however, some produce toxins, such as Shiga toxin-producing *Escherichia coli* (STEC) (McDonald, 2023). The CDC estimates that STEC causes more than 265,000 illnesses, 3,600 hospitalizations, and 30 deaths annually in the United States (Centers for Disease Control and Prevention, 2012). The most common symptoms of STEC infection in humans include severe diarrhea, stomach cramps and vomiting, and possibly bloody stools (McDonald, 2023). Infection with STEC can lead to serious clinical conditions in humans, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Arancia et al., 2019). The most common STEC is *E. coli* O157:H7, which is found in North America (Centers for Disease Control and Prevention, 2014). In the United States, serogroups O26, O45, O103, O111, O121, O145 and O157 account for the majority of STEC illnesses (Remfry et al., 2021). Pigs are considered potential hosts for some types of STEC and have also been implicated in human infections (Arancia et al., 2019).

Because pigs can carry pathogens from the pig farm to the abattoir and contaminate pork products, it is important to identify effective interventions before harvest that can reduce pathogens in market pigs and thus reduce the burden of pathogens entering the abattoir. *Lactobacillus salivarius* has been isolated from animal and human samples and can be used as a probiotic supplement for animals and humans. *Lactobacillus salivarius* L28 (Probicon) is an innovative direct-fed microbial (DFM) isolated from ground beef, which has been shown to inhibit the growth of important foodborne pathogens, such as *Salmonella* and *E. coli* O157:H7 in cattle feces (Flach et al., 2022). BIOPLUS® 2B is formulated specifically for the pig industry and is a combination of two strains of *Bacillus licheniformis* and *Bacillus subtilis* (Chr Hansen, 2023). It is used in animals to establish gastrointestinal microflora and maintain the productive performance of healthy animals (Nutriment Health, 2023).

Research Questions

1. Can BAX® System SalQuant® be used as a rapid, quantitative PCR method for *Salmonella* in pre-harvest and harvest market pig samples?
2. Can BIOPLUS® 2B be used as a pre-harvest intervention to reduce *Salmonella* and Shiga toxin-producing *E. coli* in market pigs?
3. Can Probicon L28 be used as a pre-harvest intervention to reduce *Salmonella* and Shiga toxin-producing *E. coli* in market pigs?

References:

Arancia, S., Iurescia, M., Lorenzetti, S., Stravino, F., Buccella, C., Caprioli, A., Franco, A., Battisti, A., Morabito, S., & Tozzoli, R. (2019). Detection and isolation of Shiga Toxin-producing *Escherichia coli* (STEC) strains in caecal samples from pigs at slaughter in Italy. *Veterinary Medicine and Science*, *5*(3), 462-469. https://doi.org/10.1002/vms3.175

Centers for Disease Control and Prevention. (2012). *Surveillance system overview: national Shiga toxin-producing* Escherichia coli *(STEC) surveillance*. Retrieved June 5, 2023 from https://www.cdc.gov/ncezid/dfwed/pdfs/national-stec-surveillance-overiew-508c.pdf

Centers for Disease Control and Prevention. (2014). *E. coli (Escherichia coli)*. Retrieved May 23, 2023 from https://www.cdc.gov/ecoli/general/index.html

Centers for Disease Control and Prevention. (2023). *Salmonella*. Retrieved June 6, 2023 from https://www.cdc.gov/salmonella/index.html

Chandrapati, S., & Williams, M. G. (2014). Total viable counts | Most Probable Number (MPN). In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 621-624). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00333-5

Chr Hansen. (2023). *BIOPLUS® 2B*. Retrieved June 5, 2023 from https://www.chr-hansen.com/en/animal-health/cards/product-cards/bioplus-2b

Erkmen, O. (2022). Practice 4 - Most probable number technique. In O. Erkmen (Ed.), *Microbiological analysis of foods and food processing environments* (pp. 31-37). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-323-91651-6.00042-2

Fernandez, M., Garcia, A., Vargas, D. A., & Calle, A. (2021). Dynamics of microbial shedding in market pigs during fasting and the influence of alginate hydrogel bead supplementation during transportation. *Microbiology Research*, *12*(4), 888-898. https://www.mdpi.com/2036-7481/12/4/65

Flach, M. G., Dogan, O. B., Kreikemeier, W. M., Nightingale, K. K., & Brashears, M. M. (2022). Reduction of pathogens in feces and lymph nodes collected from beef cattle fed *Lactobacillus salivarius* (L28), *Lactobacillus acidophilus* (NP51) and *Propionibacterium freudenreichii* (NP28), commercially available Direct-Fed Microbials. *Foods*, *11*(23), 3834. https://www.mdpi.com/2304-8158/11/23/3834

Heredia, N., & García, S. (2018). Animals as sources of food-borne pathogens: A review. *Animal Nutrition*, *4*(3), 250-255. https://doi.org/10.1016/j.aninu.2018.04.006

Hoy, M. A. (2013). Chapter 8 - DNA amplification by the polymerase chain reaction:molecular biology made accessible. In M. A. Hoy (Ed.), *Insect molecular genetics (Third Edition)* (pp. 307-372). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-415874-0.00008-1

Karunasagar, I., Maiti, B., & Kumar, B. K. (2018). Chapter 13 - Molecular methods to study *vbrio parahaemolyticus* and *vibrio vulnificus* from atypical environments. In V. Gurtler & J. T. Trevors (Eds.), *Methods in microbiology* (Vol. 45, pp. 387-417). Academic Press. https://doi.org/https://doi.org/10.1016/bs.mim.2018.09.001

Kralik, P., & Ricchi, M. (2017). A basic guide to Real Time PCR in microbial diagnostics: definitions, parameters, and everything. *frontiers*, *Volume 8 - 2017*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.00108/full

Marshall, K. E., MPH1; , Nguyen , T.-A., MPH1; Michael Ablan, MPH1,2; , Nichols, M. C., DVM1; , Robyn, M. P. D., Sundararaman, P. M., Whitlock, L. M., E. Wise, M. P., & A. Jhung, M. M. (2020). *Investigations of possible multistate outbreaks of* Salmonella*, Shiga toxin–producing* Escherichia coli*, and* Listeria monocytogenes *infections — United States, 2016*. Retrieved June 5, 2023 from https://www.cdc.gov/mmwr/volumes/69/ss/ss6906a1.htm

McDonald, J. V. (2023). *Shiga toxin-producing* E. coli *(STEC) infections*. Retrieved June 5, 2023 from https://www.health.ny.gov/diseases/communicable/e_coli/stec.htm

Nutriment Health. (2023). *BioPlus 2B*. Retrieved June 5, 2023 from https://nutriment.com.au/products/bioplus-2b/

Remfry, S. E., Amachawadi, R. G., Shi, X., Bai, J., Tokach, M. D., Dritz, S. S., Goodband, R. D., Derouchey, J. M., Woodworth, J. C., & Nagaraja, T. G. (2021). Shiga toxin–producing *Escherichia coli* in feces of finisher pigs: isolation, identification, and public health implications of major and minor serogroups. *Journal of Food Protection*, *84*(1), 169-180. https://doi.org/https://doi.org/10.4315/JFP-20-329

Schwartz, K. J. (2021). *Salmonellosis and* Salmonella *Infections*. Retrieved Jun 05, 2023, from https://porkgateway.org/resource/salmonellosis-and-salmonella-infections/

Scott, M. E., Mbandi, E., Buchanan, S., Abdelmajid, N., Gonzalez-Rivera, C., Hale, K. R., Jacobsen, L., Webb, J., Green, J., & Dolan, P. (2020). *Salmonella* and Shiga toxin–producing *Escherichia coli* in products sampled in the food safety and inspection service raw pork baseline study. *Journal of Food Protection*, *83*(3), 552-559. https://doi.org/https://doi.org/10.4315/0362-028X.JFP-19-360

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 and Infection*, *145*(14), 2980-2990. https://doi.org/10.1017/s0950268817002114

Chapter 2 - Literature Review

2.1 *Salmonella* background information

Salmonella was discovered and isolated by Theobald Smith in 1855 from the intestines of pigs infected with classical swine fever and was named after Dr. Daniel Elmer Salmon (Eng et al., 2015). *Salmonella* is a group of Gram-negative, non-sporogenic, prokaryotic, rod-shaped bacteria that use multiple flagella for motility (Mumy, 2014). *Salmonella* belongs to the *Enterobacteriaceae* family and is believed to have evolved from the same ancestor as *E. coli* 160-180 million years ago (Mumy, 2014). The optimum temperature for the growth of *Salmonella* is between 35°C and 37°C. (Akil et al., 2014). However, the temperature at which *Salmonella* grows is elastic, with some strains growing at temperatures as high as 54°C (Matthews et al., 2017a). When exposed to low water activity (aw) conditions, the heat resistance of *Salmonella* is greatly enhanced in comparison to high-water activity conditions (Gautam et al., 2020).

The terms "serotype" and "serovar" are often considered synonymous (Ryan et al., 2017) and the term “serotype” is used in this document. *Salmonella* is divided into two types: *Salmonella enterica*, which can infect a variety of warm-blooded animals, and *Salmonella bongori*, which only infects cold-blooded animals (Mansfield & Fox, 2019). *Salmonella enterica* is categorized into six subspecies, they are: *S. enterica* subsp. *enterica* (subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV), and *S. enterica* subsp. *indica* (subspecies VI) (Agasan et al., 2002).

According to the White-Kauffmann-Le Minor scheme, the *Salmonella* subspecies can be further divided into serotype (Löfström et al., 2016). Serotype isolation is based on antigenic

polymorphisms of lipopolysaccharide (O antigen), flagellin (H antigen): phases 1 and 2, and capsular polysaccharide (Vi antigen) (Löfström et al., 2016). Only a few serotypes have Vi antigens (Giannella., 1996). An example of an antigen formulation for serotype *Salmonella* Typhimurium based on the Kauffman-White scheme is 1,4,5, 12:i:1,2, where 1, 4, 5 and 12 are O antigen, i is phase 1 H antigen and 1 and 2 are phase 2 H antigen.

To date, 46 O antigens and 114 H antigens have been identified, representing more than 2,600 reported serotypes in different combinations (Diep et al., 2019). O antigens consist of glycan repeating units containing two to eight sugar residues (Seif et al., 2019). The final structural changes occur as a result of the order of glycan connections, the type of connections between residues, and the type of connections between O-units (Seif et al., 2019). These glycans are often O antigen-specific, and their biosynthesis has been well studied in *Salmonella*, *Escherichia*, and *Shigella* (Seif et al., 2019). H antigen is a slender, threadlike structure that is part of the flagella, and it is characterized by the protein composition within the flagella. (Centers for Disease Control and Prevention, 2022). Each O and H antigen has a unique code, and scientists determine serotype based on different combinations of O and H antigens (Centers for Disease Control and Prevention, 2022).

In clinical laboratories, strains of *S. enterica* subsp. I are usually isolated from humans and warm-blooded animals (Agasan et al., 2002). Of the 1,500 *S. enterica* subsp. I serotypes that are from 2,500 serotypes of *S*. enterica species, approximately 30 are agents of foodborne illness in humans (Gorski et al., 2022; Guard et al., 2021). Based on the structure of the O antigen, *Salmonella* is divided into 46 serogroups, of which about 70% of *Salmonella* infection in humans and animals are caused by A, B, C1, C3 and D serogroups (Zhou et al., 2014).

Typhoid serotypes (Typhi and Paratyphi A) are human adapted and cause enteric fever, but non-typhoidal serotypes (*e.g.* Typhimurium and Enteritidis) have a wide host range and primarily cause gastroenteritis (Saleh et al., 2019). Typhoid and paratyphoid are bacterial infections that affect the entire body, caused by the bacteria *Salmonella* Typhi and *Salmonella* Paratyphi, respectively (Centers for Disease Control and Prevention, 2018). Worldwide, an estimated 11 million to 21 million people are effected by typhoid fever and about 5 million people are affected by paratyphoid each year (Centers for Disease Control and Prevention, 2018). The symptoms of typhoid fever and paratyphoid fever are much alike, including weakness, stomach pain, headache, diarrhea, cough, loss of appetite, and usually associated with a sustained fever that can reach a maximum of 39-40 °C (Centers for Disease Control and Prevention, 2018). Non-typhoidal *Salmonella* is a major contributor of bacterial diarrhea worldwide, causing an estimated 153 million cases of gastroenteritis and 57,000 deaths worldwide each year (Centers for Disease Control and Prevention, 2019). The most common clinical presentation of non-typhoidal *Salmonella* infection is gastroenteritis (Centers for Disease Control and Prevention, 2019). The incubation period is generally 6-72 hours and symptoms include acute diarrhea, abdominal pain, fever, and vomiting (Centers for Disease Control and Prevention, 2019). The illness lasts for 4-7 days and most people do not need treatment (Centers for Disease Control and Prevention, 2019).

2.2 Importance of *Salmonella* Research

Salmonella *in Swine*

Salmonella infections are very common in pigs and can infect humans through contaminated pork products (Schwartz, 2021). *Salmonella* infection is also a threat to pigs of any age, and salmonellosis mainly manifests as two clinical diseases, including 1) sepsis and

pneumonia, which is usually caused by *S*. Choleraesuis, and 2) diarrhea and intestinal diseases, which is usually caused by *S*. Typhimurium and *S*. Heidelberg, among others (Schwartz, 2021). Salmonellosis caused by *S*. Choleraesuis tends to cause systemic disease in the form of septicemia and leads to high mortality in piglets. *Salmonella* in pig intestines can contaminate carcasses during slaughter, posing a potential public health risk of food poisoning (Pipestone Veterinary Services, 2018).

According to a study conducted by Pires et al. (2012), 453 cultured *Salmonella* were detected in 6,836 pig fecal samples with a prevalence of 6.6% from 3 finishing sites (Pires et al., 2012). At the pig level, 187 out of 899 pigs were found to have *Salmonella*, with a prevalence of 20.8% (Pires et al., 2012). In another study, twenty-five carcasses were selected from each of 21 commercial (N=12 for northern and N=9 for southern) pork harvesting facilities (Bessire et al., 2018). Two superficial inguinal lymph nodes were collected from each carcass, with a total 1,014 samples, among which 6.4% and 37% *Salmonella* prevalence was reported for market pigs and sows from the northern region of the United States, respectively. In the southern region of the United States, 13.0% of market pigs and 4.8% of sows were positive for *Salmonella* (Bessire et al., 2018). Another study recovered 235 isolates of *Salmonella* from 820 carcass swabs, 164 lymph nodes, and 164 cecal contents, of which prevalence was 11.5% from carcass swabs, 54.3% from cecal contents, and 31.7% from lymph node samples (Deane et al., 2022; Fernandez et al., 2021). *Salmonella* Typhimurium, *S*. Typhimurium monophasic variant, *S*. London and *S*. Derby are the most common serotypes (Deane et al., 2022; Fernandez et al., 2021).

Fecal-oral transmission is the main route of transmission between pigs, and contacting pigs with other pigs, or boots, clothing, and equipment that have been in contact with other pigs greatly increases the risk of disease transmission (Pipestone Veterinary Services, 2018).

Contaminated pigs can shed *Salmonella* Typhimurium for up to five months after recovery from clinical illness (Perle Zhitnitskiy, n.d.). *Salmonella* has the ability to adapt to new environments and survive even in the most inhospitable conditions, making it impossible to eradicate (Pipestone Veterinary Services, 2018). Wild animals, including rodents, birds, and cats can also carry *Salmonella* and contaminate pigs. *Salmonella* has also been found in animal feed and can survive in water (Agriculture and Horticulture Development Board, 2023). The concern of *Salmonella* infection in pigs because it can result in clinical disease in pigs and pigs may be infected with multiple *Salmonella* serotypes that can contaminate pork products and pose a threat to human health (Dickson et al., 2019).

Salmonella *in Pork*

According to the Food and Agriculture Organization of the United Nations (FAO), the most widely consumed meat in the world is pork (36%) and second is poultry (33%) (Food and Agriculture Organization of the United Nations, 2022). Pork consumption in the U.S. ranged from 72.64 to 53.19 pounds per person between 1961 and 2017, with the most recent data showing that the average American eats 66.18 pounds of pork per year (Christen, 2021). According to U.S. Census data and the Simmons National Consumer Survey (NHCS), 268 million Americans ate bacon in 2020, and more than 16 million of them consumed five or more pounds of bacon in a given year (Christen, 2021). The value of U.S. pork and pork products exported around the world reached a record $7.7 billion in 2020, which is up 11% from the previous year (U.S. Department of Agriculture, n.d.-b). In 2022, the United States exported more than seven billion pounds of pork worldwide (Shahbandeh, 2023).

There are many sources of human infection, and food is a major source. Animal products (beef, poultry, pork) as well as vegetables, fruits, nuts and spices are the most common sources

of *Salmonella* in the human food chain (Schwartz, 2021). In addition to poultry, including laying hens and turkeys, pigs are one of the main animals that transmit *Salmonella* to humans (Bonardi, 2017). According to an analysis of foodborne illness outbreaks in the United States, pork may be responsible for 8 to 13 percent of the estimated one million foodborne human salmonellosis cases annually (Food Safety and Inspection Service, 2022b).

A comparison of infection rates between pigs on farms and in slaughterhouses shows that infection rates tend to be lower on farms, in part due to the presence of potential and undetectable carriers of *Salmonella* (Bonardi, 2017). The pre-harvest phase refers to the period from when pigs are kept on the farm until they are loaded and transported to the slaughterhouse (European Food Safety Authority, 2006). During this period, pigs may be infected with *Salmonella* and show clinical symptoms, they may become asymptomatic carriers and shed the pathogen in their feces, or they may harbor the pathogen in several tissues such as the digestive tract, closely related lymph nodes, or tonsils (Bonardi, 2017). The harvest stage refers to the beginning of the transport of the slaughtered animal from the farm gate until the cooling of the carcass (European Food Safety Authority, 2006). During this phase, asymptomatic pigs may begin shedding *Salmonella* after leaving the farm due to stress. Collective housing, transportation, slaughterhouse feeding, rough handling of pigs during loading and unloading, high density of animals during transportation, long transportation times, poor driver skills, poor weather conditions, and feed withdrawal can all cause stress that substantially increases *Salmonella* shedding (Bonardi, 2017).

According to recent studies, the prevalence of *Salmonella* in mesenteric lymph nodes of pigs from EU countries ranges from 7.4% to 26.0%, while surveillance programs based on analysis of pig feces reported prevalence in Denmark and Estonia of 21.6% and 27.3%,

respectively. The route of contamination may be related to the pig or slaughtering environment, and feces from pigs slaughtered on the same day may contaminate the abattoir environment, including equipment such as carcass splitters and belly openers (Bonardi, 2017).

Salmonella *Outbreaks in Pork*

According to the Centers for Disease Control and Prevention estimates, in the United States, *Salmonella* causes approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths each year, and food is the main source of these illnesses (Centers for Disease Control and Prevention, 2023). In 2013, a large outbreak caused by *Salmonella* 1, 4, [5], 12: I:- occurred in Nevada and was linked to a restaurant where pork sausage was cooked and the outbreak strain was recovered. (Self et al., 2017). In another outbreak, Kapowsin Meats issued an expanded recall on August 27, 2015 of approximately 523,380 pounds of pork products potentially contaminated with *Salmonella* 1, 4,[5],12:i:-. (Centers for Disease Control and Prevention, 2015). A total of 192 patients in five states were infected with *Salmonella* 4, b[5], 12:I:- and *Salmonella* Infantis in this outbreak (Centers for Disease Control and Prevention, 2015).

In August 2021, the CDC, public health and regulatory officials in several states, and the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) investigated a multistate outbreak of prepackaged and uncured antipasto trays (Fratelli Beretta brand) that were infected with *Salmonella* Infantis and *Salmonella* Typhimurium. A total of 40 people in 17 states were infected with 14 cases of *Salmonella* Infantis and 26 cases of *Salmonella* Typhimurium (Centers for Disease Control and Prevention, 2021). The true number of people who became ill in this outbreak was likely much higher than reported and may not be limited to the states with reported illnesses (Centers for Disease Control and Prevention, 2021).

Economic Impact of Salmonella

Salmonella not only has a huge impact on human health, but also causes notable economic losses. In 2013, the Economic Research Service (ERS) of the United States Department of Agriculture (USDA-ERS) reported 1,027,561 cases, 19,339 hospitalizations, and 378 deaths of non-typhoidal *Salmonella* in the United States each year, which is estimated to cause a loss of 3,666.6 million dollars (Hoffmann, 2015). These include healthcare costs, lost wages due to time off work, and societal payments to prevent death (USDA, 2015).These economic burdens include the costs of treatment and lost productivity, as well as the costs of preventing premature deaths (Hoffmann, 2015). *Salmonella* ranks first among the 15 leading U.S. foodborne pathogens in terms of economic burden (USDA, 2015).

2.3 Shiga toxin-producing *Escherichia coli* (STEC) background information

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobe (Lim et al., 2010). The temperature range of growth is 4 to 45°C and the optimum temperature is 37°C (Albrecht, 2023). This organism was first described in 1885 by Theodor Escherich (Lim et al., 2010). Most *E. coli* strains colonize the gastrointestinal tract of humans and animals as normal bacteria and are harmless to humans (Lim et al., 2010). However, some strains have evolved into pathogenic *E. coli* by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenic islands (Lim et al., 2010). These strains can cause severe illness and are mainly transmitted to humans through contaminated foods such as undercooked ground meat products, unpasteurized milk, and vegetables contaminated with hazardous substances (World Health Oragnization, 2018).

Diarrhea, as defined by the World Health Organization, is three or more loose or liquid bowel movements per day and is a major public health problem worldwide (Darbandi et al.,

2016). The mortality rate associated with diarrhea is estimated at about 1.5 million to 2.5 million, particularly in Africa, Asia, and Latin America, and *E. coli* is considered to be a significant cause of epidemic and endemic diarrhea worldwide (Darbandi et al., 2016). Diarrheagenic *E. coli* are classified into several groups based on their virulence characteristics, pathogenicity mechanisms, clinical symptoms, and distinct O:H serotypes (Matthews et al., 2017b). *Escherichia coli* isolates are serologically distinguished according to three major surface antigens: O (somatic cell), H (flagella), and K (capsule) antigens (Matthews et al., 2017b). Because few laboratories can type the K antigen, serotyping based on the O and H antigens remains as the gold standard for *E. coli* typing. Currently, O groups numbered O1 to O188 have been defined, except that O31, O47, O67, O72, O94, and O122 are not designated (Fratamico et al., 2016; Furevi et al., 2020).

The strains of *E. coli* that cause gastroenteritis in humans can be grouped into six categories: enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and diffuse adherent (DAEC) (Mulla, 1999). Each category has different virulence genes, for example, *eae*, *stx1* and *stx2* for EHEC; *bfp* and *eae* for EPEC; *LT* and *ST* for ETEC, *AA* for EAEC, *invE* for EIEC, *stx1* and *stx2* for STEC (Darbandi et al., 2016). This document focuses on enterohemorrhagic *E. coli* (EHEC) because it causes the most serious disease (Matthews et al., 2017b).

Shiga toxin-producing *Escherichia coli* (STEC) were identified in 1977 and first associated with the clinical syndrome hemolytic uremic syndrome (HUS) in 1983 (Thorpe, 2004). Diarrhea-related HUS has been identified as a prominent factor contributing to acute kidney failure in children in the U.S. who are otherwise considered healthy (Thorpe, 2004). The main characteristic of STEC is that phage encoded *stx1* and/or *stx2* genes determine the production of Shiga toxin (Stx) (Darbandi et al., 2016). Virulent isolates of *E. coli* O157:H7 can

produce only Stx1, only Stx2, or both toxins (Lim et al., 2010). Strains producing Stx2 are known to be more virulent than strains producing Stx1 and is more often associated with hepatitis C or HUS in human infections (Lim et al., 2010). Several Stx subtypes have been identified in *E. coli* strains, including Stx1a, Stx1c, Stx1d, and Stx2a to Stx2g, with significant differences in biological activity, such as serological reactivity, receptor binding, and toxin potency (Yang et al., 2020). Strains of *Escherichia coli* that produce Stx2 are more likely to cause hemolytic uremia in children, and a study showed that 93.1% of all strains tested were found to carry the *stx2* gene but no *stx1* gene was detected (Tahamtan et al., 2010).

Not all STEC infect humans, and the small fraction of STEC that causes disease in humans are classified as EHEC (Joseph et al., 2020). All EHEC are STEC, but not all STEC are EHEC (Matthews et al., 2017b). Diseases range from mild diarrhea to bloody diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Bosilevac & Koohmaraie, 2012). *Escherichia coli* O157:H7 is the STEC most commonly associated with serious illnesses (Bosilevac & Koohmaraie, 2012). Many non-O157 EHEC bacteria have also been associated with similar diseases and disease outbreaks, including six serogroups, (O26, O111, O103, O121, O45, and O145) that are described by CDC as the cause of 71% of non-O157 EHEC diseases (Bosilevac & Koohmaraie, 2012).

The key virulence factor of EHEC is Stx, which enters the kidney through the bloodstream and leads to renal inflammation through direct toxicity and by inducing the binding of local cytokine and chemokine production, thereby destroying renal endothelial cells and blocking microvasculature (Kaper et al., 2004). This damage can lead to hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombocytopenia, and potentially fatal acute renal failure. Shiga toxin also mediates local damage to the colon, leading to bloody

diarrhea, hemorrhagic colitis, necrosis, and intestinal perforation (Kaper et al., 2004). The initial outbreak of EHEC was associated with the consumption of undercooked hamburgers (Kaper et al., 2004). Subsequently, a wide variety of foods have been linked to the illness, including sausages, unpasteurized milk, lettuce, cantaloupe, apple juice and radish sprouts (Kaper et al., 2004). In addition to Stx, EHEC contains an additional virulence factor, known as intimin, which allows EHEC to adhere tightly to the lining of the host intestinal tract during infection (Bosilevac & Koohmaraie, 2012). Initial screening for *stx*, *eae*, and O groups to identify presumptive positive sample enrichments is the basis of many commercial tests that enter the market (Bosilevac & Koohmaraie, 2012).

2.4 Importance of STEC Research

STEC in Swine

In swine, strains of STEC have been associated with edema disease (Tseng et al., 2014). Edema disease usually occurs after weaning or in young finishing pigs and is caused by the *stx2e* gene carried by STEC (Tseng et al., 2014). Shiga toxin 2e, produced in the intestinal tract of colonized pigs, is the primary cause of the clinical symptoms and pathological changes of edema disease (Fairbrother, 2023). This cytotoxin inhibits protein synthesis and leads to cell death (Fairbrother, 2023). Pigs are initially infected through contaminated environments or sows, then high levels of STEC from colonized pigs of infection spread between pigs in the same pen (Fairbrother, 2023). A study by Remfry et al. (2021) reported that a total of 178 isolates and 23 serogroups were isolated from 598 feces samples collected from ten pig flows, of which the three main serogroups recovered were O8, O86, and O121 (Remfry et al., 2021). Six *stx1a* subtypes and 152 *stex2e* were associated with these 178 STEC isolates (Remfry et al., 2021). In another study, 82 of 120 finishing pigs from three farms shed STEC at least once (Cha et al., 2018) and

E. coli O157:H7 (*stx2c* and *eae*) and O26:H11 (*stx1a* and *eae*) were isolated from two pigs, and 39 pigs carried serotype O59:H21 (*stx2e*) (Cha et al., 2018). A total of 598 fecal samples collected from finishing pigs in 10 pig streams were analyzed by real-time PCR (Remfry et al., 2020). Among these 598 fecal samples, the prevalence of *stx1*, *stx2* and *eae* was 25.9%, 65.1% and 67%, respectively, among which serogroups O121, O157 and O26 were the most dominant (Remfry et al., 2020).

Infection with STEC may lead to edema disease, which may occur independently or with post-weaning diarrhea, and treatment is often ineffective due to its sudden onset and rapid course (Fairbrother, 2023). The incidence of the disease is usually low, but the mortality rate in pigs with symptoms is high (Iowa State University, 2023). Symptoms include anorexia, ataxia, numbness and lying flat, often accompanied by paddling, and running movements (Iowa State University, 2023). Infected pigs may make abnormal vocalizations due to edema of the throat, as well as swelling of the face and eyelids, infected pigs die within hours or days, and a few may survive but usually have neurological dysfunction. (Iowa State University, 2023).

STEC in Pork

The presence of STEC in pork products is a growing concern even though STEC are commonly associated with cattle and beef (Nastasijevic et al., 2020). Over the years, the difficulty in detecting and differentiating serogroups of STEC strains has led the industry and regulators to focus primarily on the O157 serogroup (Haque et al., 2022). These strains spread from pig farms, where pigs may be asymptomatic carriers, to slaughter and boning operations, and eventually into the meat supply, potentially causing human infection (Haque et al., 2022). If the STEC strain contaminate the food chain means it poses a risk to consumers (Colello et al., 2016).

According to Rocio Colello (2016), STEC contamination originates at the farms, then transfers from pigs to carcasses during slaughter, and moves through boning rooms and markets. Because STEC are detected in pigs and pork products, pork must be cooked in accordance with the USDA-FSIS guidelines for safe consumption (Haque et al., 2022). More specifically, the internal temperature must reach 60°C/140°F for whole pork products and 71.1°C/160°F for incomplete pork products to reduce the potential risk of foodborne illness in humans (Haque et al., 2022).

STEC Outbreaks in Pork

Between July and October 2014, 119 outbreaks of *E. coli* O157:H7 infection were detected in Alberta, Canada (Lance Honish, 2017). Twenty-three patients were hospitalized, of whom, six developed HUS, and no deaths were reported (Lance Honish, 2017). Seven potential food sources were identified, and most patients reported eating foods containing pork at Asian-style restaurants in multiple geographically diverse cities in Alberta during the exposure period (Lance Honish, 2017). Contaminated pork and pork production environments, as well as improper handling of pork products, have been identified as key points in the supply chain where *E coli* O157:H7 contamination occurred, including slaughterhouse, processor, retail, and restaurant facilities (Lance Honish, 2017).

On 24 January 2004, a couple in a town in the northeastern Italian region of Venitto visited the emergency room with bloody diarrhea, severe abdominal pain, and nausea (Conedera et al., 2007). A third family member, their daughter, was suffering from mild diarrhea and did not seek medical attention (Conedera et al., 2007). According to the local health authorities, *E. coli* O157 was isolated from stool samples and the traditional salami that all three members of the family ate; therefore, the source of the pathogen was most likely pork (Conedera et al., 2007).

Public Health Impact of STEC

Symptoms of STEC infection vary, but the most common symptoms include severe diarrhea, stomach cramps and vomiting (McDonald, 2023). The incubation period is three to four days after exposure, but it can be as short as one day or as long as 10 days (Centers for Disease Control and Prevention, 2014). In some populations, especially children under five years of age, infection can lead to hemolytic uremic syndrome (HUS), which is a serious life-threatening condition that can lead to kidney damage or failure, and other serious problems that often require hospitalization; however, blood transfusions, plasmapheresis, or kidney dialysis may also be required (McDonald, 2023).

Shiga toxin-producing *E. coli* infection is a major public health problem worldwide, causing more than one million cases of illness, 128 deaths and 13,000 disability-adjusted life years each year (Food and Agriculture Organization of the United Nations & World Health Organization, 2019). According to published reports, in the United States, approximately 96,534 STEC O157 infections and 168,698 non-O157 infections each year (Woo & Palavecino, 2013). These infections result in more than 3,600 hospitalizations and 30 deaths each year (Woo & Palavecino, 2013). Epidemiological data on STEC in swine are limited, although pork products have been associated with STEC transmission (Ercoli et al., 2015).

2.5 Pre-harvest Interventions

Probiotics

The United Nations Food and Agriculture Organization (FOA)/World Health Organization (WHO) defines probiotics as "living microorganisms that, when given in sufficient quantities, are beneficial to the health of the host" (Brown & Valiere, 2004). There are increasing worry about antibiotic resistance and the use of probiotics in livestock to improve animal production

and health without the need for antibiotics (Wang et al., 2009). Lactic acid bacteria are widely used for human health benefits and can be used to prevent gastrointestinal diseases, for example, diarrhea, inflammatory bowel disease, lactose intolerance, as well as *Salmonella* and *Shigella* infections (Wang et al., 2009).

The first bacterium in pure culture was called *Bacterium lactis,* and was isolated by Joseph Lister in 1873 (Narvhus & Axelsson, 2003). Lactic acid bacteria (LAB) are Gram-positive, spore-free, catalase negative, acid tolerant, non-respiratory but oxygen-resistant, cocci or rod-shaped bacteria, that are generally non-pathogenic and considered safe or food grade (Mozzi, 2016). The growth of LAB requires fermentable carbohydrates (Narvhus & Axelsson, 2003). Lactic acid bacteria have two forms of fermentation: homofermentative, which produce 95% lactic acid from glucose, or heterofermentative, whose produce acetic acid, ethanol, carbon dioxide and lactic acid (Narvhus & Axelsson, 2003). There are several species of LAB that are now associated with food: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Azam et al., 2017). Lactic acid bacteria can inhibit pathogenic bacteria by competing for intestinal nutrients or binding sites on intestinal epithelial cells (Yang et al., 2015). Most intestinal pathogens must adhere to the intestinal epithelium before they can colonize the intestinal tract and cause disease, and some LAB strains have the capability to bind to the intestinal epithelium (Yang et al., 2015).

Bacillus species are Gram-positive, rod-shaped, spore-forming, aerobic or facultative anaerobic bacteria (Elshaghabee et al., 2017). *Bacillus licheniformis* and *Bacillus subtilis* are considered probiotics to prevent the growth of pathogenic microorganisms because they produce enzymes and secondary metabolites that inhibit the growth of pathogenic microorganisms, and

they are used to prevent *E. coli* or *Salmonella* (Romo-Barrera et al., 2021). *Bacillus* species are saprophytic bacteria commonly present in a variety of environmental conditions (Romo-Barrera et al., 2021). Most of the *Bacillus* species are not considered to be a risk to humans, but *B. cereus* and *B. anthracis* are considered pathogenic to humans, which inhibits their use as probiotics (Romo-Barrera et al., 2021). Spore-forming probiotics (SFP) can stimulate the immune system, synthesize different antibacterial drugs such as bacteriocins and enzymes, and regulate the composition of intestinal flora (Elshaghabee et al., 2017). *Bacillus* has strong tolerance and survivability in the harsh gastrointestinal environment where they are able to interact with the host internal environment by producing various antimicrobial peptides and extracellular small molecule effects (Elshaghabee et al., 2017).

Probicon L28 （Lactobacillus salivarius L28）

Lactobacillus salivarius is a probiotic supplement that has been isolated from animal and human samples and is beneficial to animal and human health (Flach et al., 2022). *Lactobacillus salivarius* L28 (hereafter referred to as Probicon or Probicon L28) was developed by food industry scientists from Texas Tech University as a probiotic that can be used alone, but also in a strategic mix, and it is available in liquid, frozen, and freeze-dried forms for different applications (Nexgen, n.d.). Probicon L28 is a new direct-fed microbial (DFM) that was isolated from ground beef, and many studies have shown that Probicon is effective in growth inhibition of *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Flach et al., 2022). Probicon L28 is incorporated into animal and pet foods as a food safety intervention (Nexgen, n.d.). Probicon L28 has been tested for efficacy against pathogens in several food matrices (chicken fat, dry dog kibble, rendered lamb meal) and on stainless steel. (Nexgen, n.d.). One

study evaluated that Probicon could reduce *Salmonella* in raw chicken fat by 2-logs in 24 hours (Nexgen, n.d.).

BIOPLUS® 2B

BIOPLUS® 2B is a probiotic product that consists of *Bacillus subtilis* and *Bacillus licheniformis* (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2016). The product has been approved for use with piglets, fattening pigs, fattening turkeys, calves, and sows two weeks before and during lactation (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2011). BIOPLUS® 2B is customized to meet the specific needs of pigs, ensuring production capacity of the sow and the performance of litters and nurseries (Chr Hansen, 2023). The product is in granular form and is applied to the diet at one pound of BIOPLUS® 2B per ton of complete feed for all species (Chr Hansen, 2023). According to a study by Alexopoulos et al. (2004), dietary supplementation of *Bacillus licheniformis* and *Bacillus subtilis* can significantly reduce the morbidity and mortality of pigs associated with *E. coli* diarrhea in weaned piglets (Alexopoulos et al., 2004).

2.6 Pathogen Control Actions

The USDA-FSIS ensures that the nation's meat, poultry, and processed egg supply is healthy, safe, and properly labeled (U.S. Department of Agriculture, n.d.-a). The USDA-FSIS develops requirements applicable to meat and poultry businesses in order to decrease the reduce the presence and quantity of pathogenic microorganisms on meat and poultry products and reduce the incidence of foodborne illness (Food Safety and Inspection Service, n.d.). Beginning in March 2012, in addition to *E. coli* O157:H7, the USDA-FSIS adopted a zero-tolerance policy for six additional serogroups, including *E. coli* O26, O45, O103, O111, O121, and O145 as

adulterants in raw beef products that are non-intact or intended for non-intact processing (U.S. Department of Agriculture, n.d.-a).

From June 1, 2017 to May 31, 2018, the USDA-FSIS conducted a baseline study to assess the occurrence of STEC and *Salmonella* in a variety of raw pork products (Scott et al., 2020). A total of 4,014 samples from slaughter and processing sites were tested for *Salmonella*, and 1,395 samples were analyzed for STEC (Scott et al., 2020). The national prevalence of *Salmonella* in raw pork products was 28.9% in shredded products, 5.3% in intact cuts, and 3.9% in incomplete cuts, while the prevalence of the top seven STEC (serogroups O157, O111, O103, O26, O121, O45, and O145) was less than 1% (Scott et al., 2020). The USDA-FSIS has proposed new performance standards for *Salmonella* in raw pork to achieve the agency's goal of reducing the number of salmonellosis causes by 25% annually (Food Safety and Inspection Service, 2022a). Producers producing more than 6,000 pounds of raw comminuted meat per day must have no more than 13 *Salmonella* positive samples per 52 samples, and producers producing more than 50,000 pounds of raw, whole, or non-whole pork slices per day must have no more than six *Salmonella* positive samples per 52 samples (Food Safety and Inspection Service, 2022a).

2.7 Enumeration Methods

Directing Plating

A direct plate count is the count of viable cells and the principle is to replicate living cells under conditions suitable for the specimen and to produce visible colonies (Libretexts, 2022). Results are typically expressed as colony-forming units per milliliter (CFU per mL) (Libretexts, 2022). Microbiologists usually count plates with 30 to 300 colonies because this range allows the number of colonies to be calculated closer to the true number of viable bacteria in a human population (Libretexts, 2022). There are two common methods for viable counts of inoculated

plates: the pour plate method and the spread plate method, and both methods require serial dilution to obtain plates with CFUs in the range of 30 to 300, which is typically achieved using a series of multiple 10-fold dilutions (Libretexts, 2022). The diluted samples are added to solid growth media, spread evenly, then incubated to form individual colonies (Austin & Pagotto, 2003). Another method is called the pour plate method and it counts the number of bacteria that can form colonies in a liquid sample that is combined with molten agar media and added to a petri dish (Tankeshwar, 2022). However, the direct plating method is limited by the volume of each plate, and if there is background flora with high growth levels on the plates, the colony counts might be affected (Chen et al., 2017).

3M™ Petrifilm™

3M™ Petrifilm™ is a pre-made culture media that contains cold water-soluble gelling agents and indicators which aids in the counting of colonies (3M, 2017a). There are many options for 3M™ Petrifilm™ standard plates, including Aerobic Count Plate, *Enterobacteriaceae* Count Plate, *E. coli*/Coliform Count Plate, Environmental *Listeria* Plate, Lactic Acid Bacteria Count Plate, etc (3M, 2021). Compared to the traditional agar method, 3M™ Petrifilm™ are ready to use, which reduces agar preparation time, while using less incubator space because of the compact size (Ledtechno, 2020). On the other hand, it has the disadvantage of having many small colonies on the edge of an overcrowded plate; thus, further sample dilution is required (Zina Saab khudhir, 2013). The countable range is often different; for example, the 3M***™*** Petrifilm™ *E coli*/Coliform count plate has a recommended upper limit of 150 colonies, while the normal plate counts in the range of 30 to 300 colonies (3M, 2017b; Libretexts, 2022).

Most Probable Number

The Most Probable Number technique, which is more commonly referred to as MPN, is a statistical methodology utilized for estimating the number of viable cells of specific microorganisms in a sample such as food or water (Integra Biosciences, 2021). The MPN technique is particularly useful when the sample contains a low population of target bacteria, and it is used when reliable viable cell counts cannot be provided by direct plate counting (Integra Biosciences, 2021). An MPN is particularly well suited for milk and water samples, as well as for samples with interfering particulate matter (Food and Drug Administration, 2020).

An MPN is performed by 1) serially diluting the sample to a level where there are no longer viable microorganisms (dilution to extinction), and 2) inoculating multiple dilutions in triplicate (at a minimum) using a suitable media for the target organism (Jha, 2016). More specifically, the MPN uses a 10-fold dilution series and then one milliliter of each dilution is inoculated into a separate tube containing broth media (Corrosionpedia, 2017). To improve accuracy, the MPN procedure uses a minimum of three dilutions, and commonly 3, 5, or even 10 tubes are used for each dilution (Mountain Empire Community College, n.d.). After incubation, positive and negative tube distributions are recorded and standardized MPN tables are used to determine the most probable number of microorganisms for each unit (*e.g.* MPN/mL) of the original sample (Mountain Empire Community College, n.d.).

The MPN is a common technique that can be used for counting total numbers of *Salmonella*, *Staphylococcus*, *Escherichia coli*, etc. (Erkmen, 2022). However, the MPN also has many disadvantages, including use of a lot of glassware and materials, and a long time to receive results; thus, this method requires a lot of labor, materials, and resources (Erkmen, 2022).

Because there are many duplicate tubes, others factors, such as the procedure of inoculation, can affect the accuracy of the results (Corrosionpedia, 2017).

Real-Time Polymerase Chain Reaction

Real-Time PCR, also known as quantitative PCR or qPCR, uses similar DNA amplification conditions as conventional PCR (Tang, 2023). The procedure is to heat the DNA to denature it, thereby separating it into two single-stranded DNA strands. Taq polymerase then uses the original DNA strands as templates to construct two new DNA strands, and each of these strands can then be used to create two new copies, and so on (National Human Genome Research Institute, 2020). However, Real-Time PCR does not require agarose gel electrophoresis and can detect PCR products in real time (Tang, 2023). The first version of PCR used gel electrophoresis to detect the PCR products after DNA amplification. This labor-intensive protocol has limitations, including inaccurate quantification, poor sensitivity, and the risk of post-PCR processing causing carry-over contamination (Löfström et al., 2015). In 1992, Higuchi et al. described the concept of real-time PCR in which the addition of a fluorescent reporter system made it possible to monitor the accumulation of PCR amplicons in real time (Higuchi et al., 1992; Löfström et al., 2015). Real-time PCR is much faster, requiring less time than conventional PCR (Ramamurthy et al., 2011). Real-time quantitative polymerase chain reaction (qPCR) is widely used in foodborne pathogen analysis and diagnosis (Löfström et al., 2015). It is a good alternative to the more time-consuming and laborious traditional culture-based method (Löfström et al., 2015).

There are two techniques designed to produce fluorescence during PCR, and are referred to as SYBR Green and Taqman (Samanthi, 2017). The SYBR Green method is based on the insertion of a nucleic acid staining dye that binds to the resulting double-stranded DNA to detect

amplification, and Taqman is a method based on a hydrolysis probe that is a double-labeled probe, and amplification is detected by degradation of the probe and release of the fluorophore by Taq polymerase (Samanthi, 2017). Fluorescence is assessed following each real-time PCR cycle, and the intensity of the fluorescence signal indicating the current quantity of DNA amplicons present in the sample at a particular moment time (Kralik & Ricchi, 2017). In the initial cycle, the fluorescence is difficult to distinguish from the background because it is too low, but as fluorescence intensity increases above detectable levels, it is proportional to the initial number of template DNA molecules in the sample (Kralik & Ricchi, 2017).

BAX® System SalQuant®

BAX® System SalQuant® is a method developed by Hygiena™ for enumerating *Salmonella* in a variety of beef, pork, poultry, environmental, and laboratory sample types (Hygiena, 2023b). The SalQuant® quantitative protocol is divided into three parts, including enrichment, PCR testing, and data analysis (Lopez, 2022). Accurate results allow for fewer repeated tests, each test is performed in a closed tube system that prevents contamination, and the BAX® System has fewer false positive results (Hygiena, 2017).

The BAX® System rapidly and rapidly detects *Salmonella* in a variety of foods, with results available in approximately 60 minutes (Hygiena, 2023a). The BAX® System uses real-time PCR quantification technology that simplifies testing methods, improves specificity and sensitivity, and supports food safety testing needs (Hygiena, 2023a). As academics and industry continue to evolve, pathogen quantification will continue to be important, and the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recognizes that simply detecting the presence or absence of *Salmonella* is not enough, rather the quantity of bacteria might also have an impact on the illness (Hygiena, 2021).

References:

3M. (2017a). *Petrifilm aerobic interpretation guide - 3M*. Retrieved June 24, 2023 from https://multimedia.3m.com/mws/media/236194O/petrifilm-aerobic-interpretation-guide.pdf

3M. (2017b). *Petrifilm coliform count plate interpretation guide - 3M*. Retrieved Jun 24, 2023 from https://multimedia.3m.com/mws/media/1715599O/3m-petrifilm-coliform-count-interpretation-guide.pdf

3M. (2021). *3M Petrifilm plates and 3M Petrifilm plate reader advanced available*. Retrieved Jun 23, 2023 from https://multimedia.3m.com/mws/media/1505478O/3m-petrifilm-plates-brochure.pdf

Agasan, A., Kornblum, J., Williams, G., Pratt, C. C., Fleckenstein, P., Wong, M., & Ramon, A. (2002). Profile of *Salmonella enterica* subsp. *enterica* (subspecies I) serotype 4,5,12:i:- strains causing food-borne infections in New York City. *Journal of Clinical Microbiology*, *40*(6), 1924-1929. https://doi.org/10.1128/jcm.40.6.1924-1929.2002

Agriculture and Horticulture Development Board. (2023). Salmonella *in pigs*. https://ahdb.org.uk/knowledge-library/salmonella-in-pigs

Akil, L., Ahmad, H. A., & Reddy, R. S. (2014). Effects of climate change on Salmonella infections. *Foodborne Pathogens and Disease*, *11*(12), 974-980. https://doi.org/10.1089/fpd.2014.1802

Albrecht, J. (2023). Escherichia coli *O157:H7 (*E Coli*)*. Retrieved May 23, 2023 from https://food.unl.edu/escherichinia-coli-o157h7-e-coli

Alexopoulos, C., Georgoulakis, I. E., Tzivara, A., Kyriakis, C. S., Govaris, A., & Kyriakis, S. C. (2004). Field evaluation of the effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine*, *51*(6), 306-312. https://doi.org/10.1111/j.1439-0442.2004.00637.x

Austin, J. W., & Pagotto, F. J. (2003). MICROBIOLOGY | detection of foodborne pathogens and their toxins. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 3886-3892). Academic Press. https://doi.org/https://doi.org/10.1016/B0-12-227055-X/00774-4

Azam, M., Mohsin, M., Ijaz, H., Tulain, U. R., Ashraf, M. A., Fayyaz, A., Abadeen, Z., & Kamran, Q. (2017). Review - lactic acid bacteria in traditional fermented Asian foods. *Pakistan Journal of Pharmaceutical Sciences*, *30*(5), 1803-1814.

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

Bosilevac, J. M., & Koohmaraie, M. (2012). Predicting the presence of non-O157 Shiga toxin-producing *Escherichia coli* in ground beef by using molecular tests for Shiga toxins,

intimin, and O serogroups. *Applied and Environmental Microbiology*, *78*(19), 7152-7155. https://doi.org/10.1128/aem.01508-12

Brown, A. C., & Valiere, A. (2004). Probiotics and medical nutrition therapy. *Nutrition in Clinical Care* *7*(2), 56-68.

Centers for Disease Control and Prevention. (2014). *E. coli (Escherichia coli)*. Retrieved May 23, 2023 from https://www.cdc.gov/ecoli/general/index.html

Centers for Disease Control and Prevention. (2015). *Multistate outbreak of multidrug-resistant* Salmonella *I 4,[5],12:i:- and* Salmonella *Infantis infections linked to pork (Final Update)*. Retrieved May 23, 2023 from https://www.cdc.gov/salmonella/pork-08-15/index.html

Centers for Disease Control and Prevention. (2018). *Typhoid fever and Paratyphoid fever*. Retrieved May 23, 2023 from https://www.cdc.gov/typhoid-fever/index.html

Centers for Disease Control and Prevention. (2019). *Salmonellosis (Nontyphoidal)*. Retrieved May 22, 2023 from https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/salmonellosis-nontyphoidal

Centers for Disease Control and Prevention. (2021). Salmonella *outbreak linked to Italian-style meats*. Retrieved May 23, 2023 from https://www.cdc.gov/salmonella/italian-style-meat-08-21/index.html

Centers for Disease Control and Prevention. (2022, September 9, 2022). *Serotypes and the importance of serotyping* Salmonella. Retrieved May 22, 2023 from

https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html

Centers for Disease Control and Prevention. (2023). *Salmonella*. Retrieved June 6, 2023 from https://www.cdc.gov/salmonella/index.html

Cha, W., Fratamico, P. M., Ruth, L. E., Bowman, A. S., Nolting, J. M., Manning, S. D., & Funk, J. A. (2018). Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* in finishing pigs: Implications on public health. *International Journal of Food Microbiology*, *264*, 8-15. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2017.10.017

Chen, Y., Pouillot, R., S. Burall, L., Strain, E. A., Van Doren, J. M., De Jesus, A. J., Laasri, A., Wang, H., Ali, L., Tatavarthy, A., Zhang, G., Hu, L., Day, J., Sheth, I., Kang, J., Sahu, S., Srinivasan, D., Brown, E. W., Parish, M., . . . Macarisin, D. (2017). Comparative evaluation of direct plating and most probable number for enumeration of low levels of *Listeria monocytogenes* in naturally contaminated ice cream products. *International Journal of Food Microbiology*, *241*, 15-22. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2016.09.021

Chr Hansen. (2023). *BIOPLUS® 2B*. Retrieved June 5, 2023 from https://www.chr-hansen.com/en/animal-health/cards/product-cards/bioplus-2b

Christen, C. (2021). *Meat consumption in the U.S. is growing at an alarming rate*. Retrieved May 23, 2023 from https://sentientmedia.org/meat-consumption-in-the-us/

Colello, R., Cáceres, M. E., Ruiz, M. J., Sanz, M., Etcheverría, A. I., & Padola, N. L. (2016). From farm to table: follow-up of Shiga toxin-producing *Escherichia coli* throughout the pork production chain in Argentina [Original Research]. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00093

Conedera, G., Mattiazzi, E., Russo, F., Chiesa, E., Scorzato, I., Grandesso, S., Bessegato, A., Fioravanti, A., & Caprioli, A. (2007). A family outbreak of *Escherichia coli* O157 haemorrhagic colitis caused by pork meat salami. *Epidemiology and Infection*, *135*(2), 311-314. https://doi.org/10.1017/s0950268806006807

Corrosionpedia. (2017). *Most Probable Number (MPN)*. Retrieved May 25, 2023 from https://www.corrosionpedia.com/definition/794/most-probable-number-mpn

Darbandi, A., Owlia, P., Bouzari, S., & Saderi, H. (2016). Diarrheagenic *Escherichia coli* pathotypes frequency in Khuzestan province of Iran. *Iranian Journal of Microbiology*, *8*(6), 352-358.

Deane, A., Murphy, D., Leonard, F. C., Byrne, W., Clegg, T., Madigan, G., Griffin, M., Egan, J., & Prendergast, D. M. (2022). Prevalence of *Salmonella* spp. in slaughter pigs and carcasses in Irish abattoirs and their antimicrobial resistance. *Irish Veterinary Journal*, *75*(1), 4. https://doi.org/10.1186/s13620-022-00211-y

Dickson, J. S., Dept. of Microbiology, C. o. A., University;, I. S., H.S. Hurd & M. H. Rostagno, Unit;, P.-H. F. S. a. E. D., National Animal Disease Center, Agricultural Research Service, USDA, & Iowa, A. (2019). Salmonella *in the Pork Production Chain*. Retrieved May 23, 2023 from https://swine.extension.org/salmonella-in-the-pork-production-chain/

Diep, B., Barretto, C., Portmann, A. C., Fournier, C., Karczmarek, A., Voets, G., Li, S., Deng, X., & Klijn, A. (2019). *Salmonella* serotyping; comparison of the traditional method to a microarray-based method and an in silico platform using whole genome sequencing data. *Frontiers in Microbiology*, *10*, 2554. https://doi.org/10.3389/fmicb.2019.02554

EFSA Panel on Additives and Products or Substances used in Animal Feed. (2011). Scientific Opinion on the safety and efficacy of BioPlus 2B (*Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750) as a feed additive for sows. *European Food Safety Authority Journal*, *9*(9), 2356. https://doi.org/https://doi.org/10.2903/j.efsa.2011.2356

EFSA Panel on Additives and Products or Substances used in Animal Feed. (2016). Safety and efficacy of BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) as a feed additive for sows, piglets, pigs for fattening, turkeys for fattening and calves. *European Food Safety Authority Journal*, *14*(9), e04558. https://doi.org/https://doi.org/10.2903/j.efsa.2016.4558

Elshaghabee, F. M. F., Rokana, N., Rohini D. Gulhane Chetan Sharma, & Panwar, H. (2017). *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Frontiers in Microbiology*, *8 - 2017*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.01490/full

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

Ercoli, L., Farneti, S., Ranucci, D., S, cuota, S., & Branciari, R. (2015). Role of verocytotoxigenic *Escherichia coli* in the swine production chain. *Italian Journal of Food Safety*. https://doi.org/https://www.pagepressjournals.org/index.php/ijfs/article/view/ijfs.2015.5156

Erkmen, O. (2022). Practice 4 - Most probable number technique. In O. Erkmen (Ed.), *Microbiological analysis of foods and food processing environments* (pp. 31-37). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-323-91651-6.00042-2

European Food Safety Authority. (2006). Opinion of the scientific panel on biological hazards (BIOHAZ) related to "Risk assessment and mitigation options of *Salmonella* in pig production". *European Food Safety Authority Journal*, *4*(4), 341. https://doi.org/https://doi.org/10.2903/j.efsa.2006.341

Fairbrother, J. M. (2023). *Edema disease in pigs*. Retrieved May 5, 2023 from https://www.merckvetmanual.com/generalized-conditions/edema-disease/edema-disease-in-pigs

Fernandez, M., Garcia, A., Vargas, D. A., & Calle, A. (2021). Dynamics of microbial shedding in market pigs during fasting and the influence of alginate hydrogel bead supplementation during transportation. *Microbiology Research*, *12*(4), 888-898. https://www.mdpi.com/2036-7481/12/4/65

Flach, M. G., Dogan, O. B., Kreikemeier, W. M., Nightingale, K. K., & Brashears, M. M. (2022). Reduction of pathogens in feces and lymph nodes collected from beef cattle fed

Lactobacillus salivarius (L28), *Lactobacillus acidophilus* (NP51) and *Propionibacterium freudenreichii* (NP28), commercially available Direct-Fed Microbials. *Foods*, *11*(23), 3834. https://www.mdpi.com/2304-8158/11/23/3834

Food and Agriculture Organization of the United Nations. (2022). *Strengthening gender-responsive climate policies and actions in the livestock sector*. Retrieved from https://www.fao.org/3/cc2998en/cc2998en.pdf

Food and Agriculture Organization of the United Nations, & World Health Organization. (2019). *Attributing illness caused by Shiga toxin-producing* Escherichia coli *(STEC) to specific foods*.

Food and Drug Administration. (2020). *BAM Appendix 2: Most Probable Number from serial dilutions*. Food and Drug Administration Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions

Food Safety and Inspection Service. (2022a). *Changes to the* Salmonella *verification testing program: proposed performance standards for* Salmonella *in raw comminuted pork and intact or non-intact pork cuts and related agency verification procedures*. Retrieved May 24, 2023 from https://www.federalregister.gov/documents/2022/02/16/2022-03301/changes-to-the-salmonella-verification-testing-program-proposed-performance-standards-for-salmonella

Food Safety and Inspection Service. (2022b). *Pork* Salmonella *performance standards risk assessment April 8, 2020 - Feb 8, 2022*. Retrieved May 22, 2023 from

https://www.fsis.usda.gov/news-events/publications/pork-salmonella-performance-standards-risk-assessment-april-8-2020-feb-8

Food Safety and Inspection Service. (n.d.). *HACCP Guidance*. Retrieved May 23 from https://www.fsis.usda.gov/inspection/compliance-guidance/haccp

Fratamico, P. M., DebRoy, C., Liu, Y., Needleman, D. S., Baranzoni, G. M., & Feng, P. (2016). Advances in molecular serotyping and subtyping of *Escherichia coli*. *Frontiers in Microbiology*, *7*, 644. https://doi.org/10.3389/fmicb.2016.00644

Furevi, A., Ståhle, J., Muheim, C., Gkotzis, S., Udekwu, K. I., Daley, D. O., & Widmalm, G. (2020). Structural analysis of the O-antigen polysaccharide from *Escherichia coli* O188. *Carbohydrate Research*, *498*, 108051. https://doi.org/https://doi.org/10.1016/j.carres.2020.108051

Gautam, B., Govindan, B. N., Gänzle, M., & Roopesh, M. S. (2020). Influence of water activity on the heat resistance of *Salmonella enterica* in selected low-moisture foods. *International Journal of Food Microbiology*, *334*, 108813. https://doi.org/10.1016/j.ijfoodmicro.2020.108813

Giannella., R. A. (1996). Chapter 21 *Salmonella*. In B. S (Ed.), *Medical microbiology. 4th edition*.

Gorski, L., Liang, A. S., Walker, S., Carychao, D., Aviles Noriega, A., Mandrell, R. E., & Cooley, M. B. (2022). *Salmonella enterica* serovar diversity, distribution, and prevalence in public-access waters from a central California coastal leafy green-growing region from

2011 to 2016. *Applied and Environmental Microbiology*, *88*(3), e0183421. https://doi.org/10.1128/aem.01834-21

Guard, J., Rivers, A. R., Vaughn, J. N., Rothrock, M. J., Jr., Oladeinde, A., & Shah, D. H. (2021). AT homopolymer strings in *Salmonella enterica* subspecies I contribute to speciation and serovar diversity. *Microorganisms*, *9*(10). https://doi.org/10.3390/microorganisms9102075

Haque, M., Bosilevac, J. M., & Chaves, B. D. (2022). A review of Shiga-toxin producing *Escherichia coli* (STEC) contamination in the raw pork production chain. *International Journal of Food Microbiology*, *377*, 109832. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2022.109832

Higuchi, R., Dollinger, G., Walsh , P. S., & Robert, G. (1992). Simultaneous amplification and detection of specific DNA sequences. https://doi.org/https://doi.org/10.1038/nbt0492-413

Hoffmann, S. (2015). *Quantifying the impacts of foodborne illnesses*. Retrieved May 22, 2023 from https://www.ers.usda.gov/amber-waves/2015/september/quantifying-the-impacts-of-foodborne-illnesses/

Hygiena. (2017). *Precise and powerful molecular pathogen detection*. Retrieved Jun 17, 2023 from https://www.hygiena.com/wp-content/uploads/2021/04/MBFB-BAX-System-Q7-Brochure-EN.pdf

Hygiena. (2021). *Pathogen quantification – a new problem-solving innovation*. Retrieved Jun 25, 2023 from https://www.rapidmicrobiology.com/news/pathogen-quantification-a-new-problem-solving-innovation

Hygiena. (2023a). *BAX® System Q7*. Retrieved May 26, 2023 from https://www.hygiena.com/food-safety-solutions/pathogen-detection/bax-system-q7/

Hygiena. (2023b). *BAX® System SalQuant™ receives the IAFP Food Safety Innovation Award*. Retrieved June 2, 2023 from https://www.hygiena.com/bax-system-salquant-receives-the-iafp-food-safety-innovation-award/

Integra Biosciences. (2021). *Rapid and precise dilutions for the Most Probable Number test procedure with DOSE IT*. Retrieved May 25, 2023 from https://www.integra-biosciences.com/japan/en/applications/rapid-and-precise-dilutions-most-probable-number-test-procedure-dose-it

Iowa State University. (2023). *Edema disease*. https://vetmed.iastate.edu/vdpam/FSVD/swine/index-diseases/edema-disease

Jha, S. N. (2016). Chapter 4 - Basic detection techniques. In S. N. Jha (Ed.), *Rapid detection of food adulterants and contaminants* (pp. 107-123). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-420084-5.00004-4

Joseph, A., Cointe, A., Mariani Kurkdjian, P., Rafat, C., & Hertig, A. (2020). Shiga toxin-associated hemolytic uremic syndrome: A narrative review. *Toxins*, *12*(2), 67. https://www.mdpi.com/2072-6651/12/2/67

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, *2*(2), 123-140. https://doi.org/10.1038/nrmicro818

Kralik, P., & Ricchi, M. (2017). A basic guide to Real Time PCR in microbial diagnostics: definitions, parameters, and everything. *frontiers*, *Volume 8 - 2017*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.00108/full

Lance Honish, M., 2; Nisha Punja1,2; Sarah Nunn1,2; Dale Nelson1,2; Nyall Hislop, MSc1,2; Garth Gosselin1,2; Natisha Stashko, PhD2,3; Darlene Dittrich2,3 (2017). Escherichia coli *O157:H7 infections associated with contaminated pork poducts — Alberta, Canada, July–October 2014*. https://www.cdc.gov/mmwr/volumes/65/wr/mm6552a5.htm#contribAff

Ledtechno. (2020). *The advantages of using the 3M Petrifilm*. Retrieved May 25, 2023 from https://www.ledtechno.be/en/news-events/the-benefits-of-using-the-3m-petrifilm

Libretexts. (2022). *9.1 How microbes grow*. https://bio.libretexts.org/Bookshelves/Microbiology/Microbiology_(OpenStax)/09%3A_Microbial_Growth/9.01%3A_How_Microbes_Grow

Lim, J. Y., Yoon, J., & Hovde, C. J. (2010). A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *Journal of Microbiology and Biotechnology*, *20*(1), 5-14. https://www.ncbi.nlm.nih.gov/pubmed/20134227

Löfström, C., Hansen, T., Maurischat, S., & Malorny, B. (2016). *Salmonella*: Salmonellosis. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 701-705). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384947-2.00607-3

Löfström, C., Josefsen, M. H., Hansen, T., Søndergaard, M. S. R., & Hoorfar, J. (2015). 9 - Fluorescence-based real-time quantitative polymerase chain reaction (qPCR) technologies for high throughput screening of pathogens. In A. K. Bhunia, M. S. Kim, & C. R. Taitt (Eds.), *High Throughput Screening for Food Safety Assessment* (pp. 219-248). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-0-85709-801-6.00009-5

Lopez, R. B. (2022). *Development and implementation of standardized microbial quantification-based procedures for process control in pork operations* Texas Tech University].

Mansfield, K. G., & Fox, J. G. (2019). Chapter 16 - Bacterial Diseases. In R. Marini, L. Wachtman, S. Tardif, K. Mansfield, & J. Fox (Eds.), *The common marmoset in captivity and biomedical research* (pp. 265-287). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-811829-0.00016-9

Matthews, K., Kniel, K. E., & Montville, T. J. (2017a). 14. *Salmonella* Species. In *Food Microbiology - An Introduction (4th Edition)* (pp. 246). American Society for Microbiology (ASM). https://app.knovel.com/hotlink/pdf/id:kt011EDR31/food-microbiology-an/salmonella-species

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

McDonald, J. V. (2023). *Shiga toxin-producing* E. coli *(STEC) infections*. Retrieved June 5, 2023 from https://www.health.ny.gov/diseases/communicable/e_coli/stec.htm

Mountain Empire Community College. (n.d.). *Lesson 10: Physical and chemical control of microbes*. Retrieved May 25, 2023 from https://water.mecc.edu/courses/Env108/lesson10_2.htm

Mozzi, F. (2016). Lactic acid bacteria. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 501-508). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384947-2.00414-1

Mulla, Z. (1999). *E. coli*: Serotypes other than O157:H7.

Mumy, K. L. (2014). *Salmonella*. In P. Wexler (Ed.), *Encyclopedia of Toxicology (Third Edition)* (pp. 211-212). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-386454-3.00537-6

Narvhus, J. A., & Axelsson, L. (2003). Lactic acid bacteria. In B. Caballero (Ed.), *Encyclopedia of food sciences and nutrition (Second Edition)* (pp. 3465-3472). Academic Press. https://doi.org/https://doi.org/10.1016/B0-12-227055-X/00673-8

Nastasijevic, I., Schmidt, J. W., Boskovic, M., Glisic, M., Kalchayanand, N., Shackelford, S. D., Wheeler, T. L., Koohmaraie, M., & Bosilevac, J. M. (2020). Seasonal prevalence of Shiga toxin-producing *Escherichia coli* on pork carcasses for three steps of the harvest process at two commercial processing plants in the United States. *Applied and Environmental Microbiology*, *87*(1), e01711-01720. https://doi.org/doi:10.1128/AEM.01711-20

National Human Genome Research Institute. (2020). *Polymerase chain reaction (PCR) fact sheet*. National Human Genome Research Institute. Retrieved May 25, 2023 from

https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet

Nexgen. (n.d.). *Probicon L28 helps reduce pathogens that cause illness*. Retrieved May 23, 2023 from http://www.nexgenprobiotics.com/pet-food

Perle Zhitnitskiy, D., MSpVM. (n.d.). Salmonella *Typhimurium*. Retrieved May 23, 2023 from https://open.lib.umn.edu/swinedisease/chapter/salmonella-typhimurium/

Pipestone Veterinary Services. (2018). Salmonella *Infection in Pigs*. Retrieved May 23, 2023 from https://pipevet.com/salmonella-in-pigs

Pires, A. F. A., Funk, J. A., & Bolin, C. A. (2012). Longitudinal study of *Salmonella* shedding in naturally infected finishing pigs. *Epidemiology and Infection*, *141*(9), 1928-1936. https://doi.org/10.1017/S0950268812002464

Ramamurthy, M., Alexander, M., Aaron, S., Kannangai, R., Ravi, V., Sridharan, G., & Abraham, A. M. (2011). Comparison of a conventional polymerase chain reaction with real-time polymerase chain reaction for the detection of neurotropic viruses in cerebrospinal fluid samples. *Indian Journal of Medical Microbiology*, *29*(2), 102-109. https://doi.org/10.4103/0255-0857.81777

Remfry, S. E., Amachawadi, R. G., Shi, X., Bai, J., Tokach, M. D., Dritz, S. S., Goodband, R. D., Derouchey, J. M., Woodworth, J. C., & Nagaraja, T. G. (2021). Shiga toxin–producing *Escherichia coli* in feces of finisher pigs: isolation, identification, and public health implications of major and minor serogroups. *Journal of Food Protection*, *84*(1), 169-180. https://doi.org/https://doi.org/10.4315/JFP-20-329

Remfry, S. E., Amachawadi, R. G., Shi, X., Bai, J., Woodworth, J. C., Tokach, M. D., Dritz, S. S., Goodband, R. D., DeRouchey, J. M., & Nagaraja, T. G. (2020). Polymerase chain reaction-based prevalence of serogroups of *Escherichia coli* known to carry Shiga toxin genes in feces of finisher pigs. *Foodborne Pathogens and Disease*, *17*(12), 782-791. https://doi.org/10.1089/fpd.2020.2814

Romo-Barrera, C. M., Castrillón-Rivera, L. E., Palma-Ramos, A., Castañeda-Sánchez, J. I., & Luna-Herrera, J. (2021). *Bacillus licheniformis* and *Bacillus subtilis*, probiotics that induce the formation of macrophage extracellular traps. *Microorganisms*, *9*(10). https://doi.org/10.3390/microorganisms9102027

Ryan, M. P., O'Dwyer, J., & Adley, C. C. (2017). Evaluation of the complex nomenclature of the clinically and veterinary significant pathogen *Salmonella*. *BioMed Research International*, *2017*, 1-6. https://doi.org/10.1155/2017/3782182

Saleh, S., Van Puyvelde, S., Staes, A., Timmerman, E., Barbé, B., Jacobs, J., Gevaert, K., & Deborggraeve, S. (2019). *Salmonella* Typhi, Paratyphi A, Enteritidis and Typhimurium core proteomes reveal differentially expressed proteins linked to the cell surface and pathogenicity. *PLOS Neglected Tropical Diseases*, *13*(5), e0007416. https://doi.org/10.1371/journal.pntd.0007416

Samanthi. (2017). *Difference between SYBR Green and Taqman*. Retrieved May 27, 2023 from https://www.differencebetween.com/difference-between-sybr-green-and-vs-taqman/

Schwartz, K. J. (2021). *Salmonellosis and* Salmonella *Infections*. Retrieved Jun 05, 2023 from https://porkgateway.org/resource/salmonellosis-and-salmonella-infections/

Scott, M. E., Mbandi, E., Buchanan, S., Abdelmajid, N., Gonzalez-Rivera, C., Hale, K. R., Jacobsen, L., Webb, J., Green, J., & Dolan, P. (2020). *Salmonella* and Shiga toxin–producing *Escherichia coli* in products sampled in the food safety and inspection service raw pork baseline study. *Journal of Food Protection*, *83*(3), 552-559. https://doi.org/https://doi.org/10.4315/0362-028X.JFP-19-360

Seif, Y., Monk, J. M., Machado, H., Kavvas, E., & Palsson, B. O. (2019). Systems biology and pangenome of *Salmonella* O-antigens. *mBio*, *10*(4), e01247-01219. https://doi.org/doi:10.1128/mBio.01247-19

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 and Infection*, *145*(14), 2980-2990. https://doi.org/10.1017/s0950268817002114

Shahbandeh, M. (2023). *U.S. total pork imports and exports 2006-2023*. Retrieved May 23, 2023 from https://www.statista.com/statistics/194708/us-total-pork-imports-and-exports-since-2001/

Tahamtan, Y., Hayati, M., & Namavari, M. (2010). Prevalence and distribution of the *stx, stx* genes in Shiga toxin producing *E. coli* (STEC) isolates from cattle. *Iranian Journal of Microbiology*, *2*(1), 8-13.

Tang, J. Y. H. (2023). Chapter 38 - Detection of Microbiological Hazards. In V. Andersen, H. Lelieveld, & Y. Motarjemi (Eds.), *Food safety management (Second Edition)* (pp. 835-850). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-820013-1.00002-4

Tankeshwar, A. (2022). *Pour plate method: procedure, uses, (dis) advantages*. Retrieved June 21, 2023 from https://microbeonline.com/pour-plate-method-principle-procedure-uses-dis-advantages/

Thorpe, C. M. (2004). Shiga toxin—producing *Escherichia coli* infection. *Clinical Infectious Diseases*, *38*(9), 1298-1303. https://doi.org/10.1086/383473

Tseng, M., Fratamico, P. M., Manning, S. D., & Funk, J. A. (2014). Shiga toxin-producing *Escherichia coli* in swine: the public health perspective. *Animal Health Research Reviews*, *15*(1), 63-75. https://doi.org/10.1017/s1466252313000170

U.S. Department of Agriculture. (n.d.-a). *Health and Safety*. Retrieved May 23, 2023 from https://www.usda.gov/topics/health-and-safety

U.S. Department of Agriculture. (n.d.-b). *Pork 2020 export highlights*. Retrieved May 23, 2023 from https://www.fas.usda.gov/pork-2020-export-highlights

USDA, E. R. S. (2015). *Foodborne illnesses caused by* Salmonella *cost the U.S. an estimated $3.7 billion annually*. Retrieved May 23, 2023 from https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail/?chartId=77908

Wang, Y., Cho, J. H., Chen, Y. J., Yoo, J. S., Huang, Y., Kim, H. J., & Kim, I. H. (2009). The effect of probiotic BioPlus 2B® on growth performance, dry matter and nitrogen digestibility and slurry noxious gas emission in growing pigs. *Livestock Science*, *120*(1), 35-42. https://doi.org/https://doi.org/10.1016/j.livsci.2008.04.018

Woo, J. S., & Palavecino, E. L. (2013). Four-year experience with simultaneous culture and Shiga toxin testing for detection of Shiga toxin-producing *Escherichia coli* in stool samples. *Journal of Clinical Microbiology*, *51*(3), 985-987. https://doi.org/10.1128/jcm.02529-12

World Health Oragnization. (2018). *E.coli*. Retrieved May 23, 2023 from https://www.who.int/news-room/fact-sheets/detail/e-coli

Yang, F., Hou, C., Zeng, X., & Qiao, S. (2015). The use of lactic acid bacteria as a probiotic in swine diets. *Pathogens*, *4*(1), 34-45. https://doi.org/10.3390/pathogens4010034

Yang, X., Bai, X., Zhang, J., Sun, H., Fu, S., Fan, R., He, X., Scheutz, F., Matussek, A., & Xiong, Y. (2020). *Escherichia coli* strains producing a novel Shiga toxin 2 subtype circulate in China. *International Journal of Medical Microbiology*, *310*(1), 151377. https://doi.org/https://doi.org/10.1016/j.ijmm.2019.151377

Zhou, X., Liu, B., Shi, C., & Shi, X. (2014). Mutation of a *Salmonella* serogroup-C1-specific gene abrogates O7-antigen biosynthesis and triggers NaCl-dependent motility deficiency. *PLOS ONE*, *9*(9), e106708. https://doi.org/10.1371/journal.pone.0106708

Zina Saab khudhir, N. H. N., Drgham.H.Yousif. (2013). Comparison between the Petrifilm™ and the conventional methods for enumerating aerobic bacteria and *E. coli* in locally produced soft chees in Baghdad. In.

Chapter 3 - *Salmonella* Quantification Utilizing SalQuant® and the BAX® System for Swine and Pork Samples

Abstract

Since *Salmonella* is present on pig farms and in the intestines of pigs, a variety of pre-harvest and harvest sample types can be used to quantify *Salmonella* in pigs. One possible source of *Salmonella* contamination in humans is pork and pork products. Traditional *Salmonella* quantification methods are often time-consuming, so it has become particularly important to identify a fast and effective detection and quantification method for *Salmonella* in the pork industry. Therefore, this study developed and validated SalQuant® System for rapid quantification of *Salmonella* in boot covers, ropes, and feces (pre-harvest pig environment) and spleens (collected at harvest). Samples that screened negative for *Salmonella* were inoculated with *Salmonella* Typhimurium 14028 at concentrations of 0-4 or 0-8 $\log_{10}$ CFU/g (mL) and analyzed by the BAX® System Real-Time Assay for *Salmonella* after incubation at 42°C for 0 (5 to 8 $\log_{10}$ CFU/g samples only), 6, 8, and 10 hours. For each sample type, a total of three biological replicates and 5 technical replicates were completed. Quantification using most probable number (MPN) was also completed for one biological replicate as a comparison. Following data collection, a linear fit equation was calculated for each incubation time. Thresholds of $R^2 > 0.80$ and log RMSE < 0.60 were used to determine the incubation time that quantifies *Salmonella* most accurately. *Salmonella* concentrations generated by MPN and SalQuant® for one biological replication were compared using a paired t-test for each sample type, and the two methods did not vary ($P>0.05$), except spleens ($P=0.0246$). These data suggest that SalQuant® can be used as a rapid, PCR-based quantification method for quantifying

Salmonella from a variety of pig samples; however, additional research is needed to validate the accuracy of using the curve developed for spleens.

3.1 Introduction

Based on Centers for Disease Control and Prevention (CDC) estimates, *Salmonella* is a zoonotic disease that causes about 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the United States every year (Centers for Disease Control and Prevention, 2019). Pigs can be infected with *Salmonella*, which can lead to economic losses for swine farms but also public health problems. *Salmonella* is a natural inhabitant of the swine gastrointestinal tract and can be found in the environment of swine farms. *Salmonella* infection in pigs is a concern because this can 1) cause clinical diseases, such as septicemia and enterocolitis (Kahya & Demirbilek, 2017), and 2) be a potential source of human disease (Dickson et al., 2019). Intensive farming causes the disease to easily spread from animal to animal (Matthews et al., 2017) and transmission of *Salmonella* from pig to pig occurs mainly via the fecal-oral route (Boyen et al., 2008).

The United States Food Safety and Inspection Service (USDA-FSIS) announced, and is seeking comments on their proposed performance standards for reducing *Salmonella* in raw pork meal and raw whole or partially whole pork cuts (Food Safety and Inspection Service, 2022). The USDA-FSIS will continue to aim for a 25 percent reduction in salmonellosis in order to achieve the "Healthy People by 2030" goal, under which the target remains unchanged (Food Safety and Inspection Service, 2022). To meet the 25% reduction target, approximately 8,300 and 21,600 *Salmonella* illnesses per year from raw comminuted pork and raw whole or incomplete pork cuts would be reduced, respectively (Food Safety and Inspection Service, 2022). In order to reach the "Healthy People by 2030" goal, we need a robust food testing methodology that incorporates real time Polymerase Chain Reaction (PCR).

Real-time fluorescence quantitative PCR is a technique used to quantitatively determine the presence of target sequences or genes in samples. In real-time PCR, the accumulation of amplified products is observed with a fluorescent reporter and reaction progress is monitored after each cycle. Thus, an increase in fluorescence can be detected by doubling the number of PCR products in each PCR cycle. The defined signal strength must be exceeded until the product is large enough to detect fluorescence over the background. The number of cycles in which sufficient amplification products have accumulated to exceed this threshold is called the CT value. (Lopez, 2022).

The most probable number (MPN) technique dates back to the early days of microbiology and is a statistical method based on probability theory. The sample is continuously diluted to the point where there are no more living microbes and multiple dilutions are inoculated into a suitable broth growth medium. Some identifiable characteristics, such as acid production or development of turbidity, are used to indicate growth. The arrangement of positive tests in the comparative statistical probability table is used to determine the bacteria concentration in the initial sample. The more replication tubes used, the better the accuracy of estimating bacterial population size. (Bari & Yeasmin, 2022).

Although the MPN method is still widely used, it is labor-intensive, costly, and requires a lot of laboratory space, all of which are difficult when dealing with a large number of samples. Real-time PCR is an efficient, rapid, and accurate detection method. The advantages of real-time PCR include sensitivity and the ability to 1) measure DNA concentrations over a wide range, 2) process multiple samples simultaneously, and 3) provide information more quickly (Hoy, 2013).

Recognizing that *Salmonella* is a concern in the pork industry and the need exists for rapidly quantifying populations from a variety of sample types, the objective of this study was to

use the BAX® System to develop SalQuant® for the rapid quantification of *Salmonella* from pig pre-harvest (boot covers, ropes, feces) and harvest (spleens) samples.

3.2 Materials and Methods

3.2.1 Experimental Design

The experimental design for boot covers, ropes, and spleens included three biological replicates at each inoculation level, whereby samples screened as negative for *Salmonella* were inoculated with *Salmonella* Typhimurium ATCC 14028 at concentrations of 0.00-4.00 $\log_{10}$ CFU/g (mL), analyzed at three enrichment timepoints (6, 8, 10 h), with five technical replicates analyzed per biological replicate. For pig feces, this experimental design included three biological replicates, whereby samples screened as negative for *Salmonella* were inoculated with *Salmonella* Typhimurium ATCC 14028 at concentrations of 0.00-8.00 $\log_{10}$ CFU/g, analyzed at four enrichment timepoints, with five technical replicates (PCR lysates) analyzed per biological replicate at four enrichment timepoints: 0 (5-8 $\log_{10}$ CFU/g only), 6, 8, and 10 h (Figure 1).

SalQuant® timepoints were analyzed using JMP® v. 15 to calculate log RMSE and R^2 to determine the timepoint most effective for enumerating *Salmonella* in pork production boot covers, ropes, spleen, and pig feces.

3.2.2 Sample Collection

Boot Covers

Each fresh boot cover (N=30) (Safetrack High Traction, QC Supply, Schuyler, NE) was placed into a Whirl-Pak bag (Nasco; Fort Atkinson, WI), massaged with ten mL buffered peptone water (BPW; Hygiena, Camarillo, CA) to moisten, then placed over boots. Walking at a normal pace throughout pens and the aisles of a market pig finishing barn, boot covers were worn to generate a boot cover that represents a finishing barn microbiome. After 200 steps, each

boot cover was placed into a separate Whirl-Pak bag. The boot covers were stored on ice and sent to the Food Safety and Defense Laboratory at Kansas State University (FSDL).

Ropes

Rope samples (N=35) with three to four inches length were procured from a large commercial USDA-FSIS inspected pork producer, where they were hung in lairage pens for greater than 30 minutes to allow for pigs to chew, placed into Ziploc (Ziploc, San Diego, CA) sample bags, and shipped on ice to the FSDL.

Spleens

Spleen samples (N=35) were procured from a large commercial USDA-FSIS inspected pork producer, where they were collected from market pigs at harvest, placed into Ziploc (Ziploc) sample bags, and shipped on ice to the FSDL.

Pig Feces

Freshly voided pig feces (N=35) were collected from the ground of market pig pens by scooping the top layer using a fresh wooden applicator stick into a Whirl-Pak sample bag until approximately 15 to 20 grams was collected into 35 individual containers. After collection, the feces were stored on ice and sent to the FSDL.

3.2.3 Inoculation Procedures

Salmonella Typhimurium ATCC 14028 was removed from the freezer at -80°C, streaked to trypticase soy agar (TSA; BD BioSciences; Franklin Lakes, NJ), and incubated at 37°C for 24 hours. After incubation, a typical colony was selected and transferred to 9 mL brain heart infusion broth (BHI; BD Biosciences; Franklin Lakes, NJ) in triplicate (three separate colonies into three separate tubes) and incubated at 37°C for 24 hours. From each tube, one hundred μL of the overnight culture were transferred to 9 mL BHI broth and incubated at 37 C for 18 hours.

The overnight culture was serially diluted in 9 mL BPW tubes, and the concentration of each BPW tubes was confirmed by spread plating in duplicate onto TSA (BD Biosciences) agar prior to incubation at 37°C for 24 hours. Typical colonies were counted per plate, averaged, and reported as log_{10} CFU/mL.

3.2.4 Sample Processing and Curve Development

Boot Covers

Boot covers (N = 30) were placed in sterile Whirl-Pak bags with 100 mL of BPW, and homogenized by a Smasher (bioMérieux, Marcy-l'Étoile, France) for 1 minute. One mL of the homogenate was added to a sterile tube, incubated for 18 – 24 h at 42˚C and screened for *Salmonella* using the BAX® System Real-Time Assay for *Salmonella* (Hygiena, Camarillo, CA). If the BAX® System results were positive, the sample was not used. Fifteen negative samples were inoculated with *Salmonella* Typhimurium ATCC 14028 at concentrations of 0.00 – 4.00 log_{10} CFU/mL (three boot covers per dilution) with one uninoculated sample used as a control. Thirty µL of inoculated boot cover slurry were transferred into a new sterile Whirl-Pak bag with 30 mL of pre-warmed (42℃) BAX MP (Hygiena, Camarillo, CA) with 1 mL/1 L of Quant Solution (QS; Hygiena, Camarillo, CA), homogenized by hand for 30 s, and incubated at 42°C for 6, 8, and 10 h. At each timepoint, the samples were removed from the incubator and analyzed using the BAX® System Real-Time Assay for *Salmonella*. Cycle threshold values from the BAX® System result reports were recorded for each timepoint and utilized for linear regression.

Ropes

Each rope (N = 35) was placed into a Whirl-Pak bag with 300 mL BAX MP media containing 1 mL/1 L of QS and homogenized by hand for 1 minute. Ten mL of the homogenate were transferred to sterile tubes, incubated for 18-24 h at 42˚C, and screened for *Salmonella*

using the BAX® System Real-Time Assay for *Salmonella*. If the BAX® System results were positive, the sample was not used. Fifteen negative samples were inoculated with *Salmonella* Typhimurium ATCC 14028 at concentrations of 0.00-4.00 log_{10} CFU/mL (three ropes per dilution) with one uninoculated sample used as a control. Samples were homogenized by hand for 30 s and incubated at 42°C for 6, 8, and 10 h. At each timepoint, the samples were removed from the incubator and analyzed using the BAX® System Real-Time Assay for *Salmonella*. Cycle threshold values from the BAX® System result reports were recorded for each timepoint and utilized for linear regression.

Spleen

Each spleen (N = 35) was cut by sterilized scissors, weighed to 25g, boiled in water for three seconds, then placed into a filtered Whirl-Pak bag. Each sample was combined with 100 mL of BPW prior to being homogenized by a Smasher for 1 minute and incubated for 18-24 hours at 42˚C. Samples were screened for *Salmonella* using the BAX® System Real-Time Assay. If the BAX® System results were positive, the sample was not used. Fifteen negative samples were inoculated with inoculum at concentrations of 0.00-4.00 log_{10} CFU/mL (three spleens per dilution) with one uninoculated sample used as a control. Thirty µL of inoculated spleen slurry were transferred into a new sterile Whirl-Pak bag with 30 mL of pre-warmed (42℃) BAX MP containing 1mL/1 L QS, homogenized by hand for 30 s, and incubated at 42°C for 6, 8, and 10 h. At each timepoint, the samples were removed from the incubator and analyzed using the BAX® System Real-Time Assay for *Salmonella*. Cycle threshold values from the BAX® System result reports were recorded for each timepoint and utilized for linear regression.

Pig Feces

Pig fecal samples (N = 35) were weighed (10 g) into a sterile Whirl-Pak bag, combined with 90 mL of pre-warmed (42˚C) BAX MP media containing 0.5 mL/1 L of QS, and homogenized by a Smasher for 1 minute. Ten mL of the homogenate were added to a sterile tube, incubated for 18 – 24 h at 42˚C, and screened for *Salmonella* utilizing the BAX® System Real Time Assay for *Salmonella*. If the BAX® System results were positive, the sample was not used. For screened negative samples, 10 mL of each fecal slurry were transferred to a new Whirl-Pak bag. Fifteen negative samples were inoculated with *Salmonella* Typhimurium ATCC 14028 at concentrations of 0.00-4.00 $\log_{10}$ CFU/g (low-level concentration) (three fecal samples per dilution) , homogenized by hand for 30 seconds, and one negative sample was uninoculated as a control sample. To each slurry, 10 mL of pre-warmed (42°C) BAX MP with 0.5 mL/1 L of QS were added. The samples were incubated at 42°C for 6, 8, and 10 h. At each timepoint, samples were removed from the incubator and analyzed using the BAX® System Real-Time Assay for *Salmonella*. The additional 12 negative samples were inoculated at concentrations of 5.00-8.00 $\log_{10}$ CFU/g (high-level concentration) (three fecal samples per dilution). Each sample was combined with 10 mL of pre-warmed (42°C) BAX MP containing 0.5 mL/1L of QS and immediately analyzed using the BAX® System Real-Time Assay for *Salmonella* (no incubation; 0 hours). Cycle threshold values from the BAX® System result reports were recorded for each timepoint and utilized for linear curve development for low-level and high-level samples.

3.2.5 Curve Verification

Curve verification was conducted for each sample type. One biological replicate from each inoculation concentration was used to perform a 3×5 MPN for negative control samples, 0.0, 1.0, 2.0, 3.0, and 4.0 $\log_{10}$ CFU/mL (g) level samples. For pig feces samples, a 3×9 MPN was used for the high-level (5-8 $\log_{10}$ CFU/g) concentration samples. The MPN tubes were incubated for

24 h at 37°C. After incubation, each tube was analyzed using the BAX® System Real-Time Assay for *Salmonella,* with one lysate per sample. The sequence of positive and negative MPN tubes was recorded and compared to the MLG 2.05 MPN tables (U.S. Department of Agriculture, 2014) to determine *Salmonella* concentration with a 95% confidence interval, and these data were transformed into $\log_{10}$ MPN/mL.

3.2.6 Statistical Analysis

For each sample type, JMP® v. 15 (SAS Institute, Cary, NC) was used to calculate linear fit equations for each incubation timepoint using cycle threshold values obtained for each inoculation concentration. An $R^2 > 0.80$ and log RMSE < 0.60 were used as thresholds to determine which incubation time quantifies *Salmonella* most effectively. Microsoft Excel (Microsoft, Redmond, WA) was used to calculate the mean *Salmonella* concentration with a 95% confidence interval for all technical replicates from biological replication one. A paired t-test (GraphPad Prism 9.0, La Jolla, California) was used to compare the *Salmonella* concentrations with 95% confidence interval generated by MPN from biological replication one with those of SalQuant® from biological replication one and a threshold of $P<0.05$ was used to determine statistical significance.

3.3 Results

3.3.1 Curve Development

The CT values were evaluated for each matrix at each time point, and linear-fit curve equations, R^2 and root mean square errors (RMSE) were established. Linear equations were used to estimate pre-enriched *Salmonella* levels to simulate logarithmic growth of bacteria. The R^2 value evaluated the percentage change in the dependent variable, explained by the linear model, which can be observed when changes in CT values depend on enrichment time and known

bacterial inoculation levels. The log RMSE assessed the standard deviation of the data and described how well the CT value estimated *Salmonella* concentration. SalQuant® equations were based on R^2 (> 0.80), the log RMSE (< 0.60) and enumerable range 0.00-4.00 $\log_{10}$ CFU/ml (g) for boot covers, spleen, and ropes; 0.00-8.00 $\log_{10}$ CFU/g for pig feces samples.

The statistical parameters estimated by all linear fit equations for enrichment times are R^2 between 0.81-0.91 and log RMSE between 0.40-0.62 (Table 1). The enumerable range is the range where SalQuant® provides accurate *Salmonella* estimates for each matrix boot covers and spleen (0.00-4.00 Log_{10} CFU/g (mL)), ropes (2.00-4.00 Log_{10} CFU/mL), and pig feces (0.00-8.00 Log_{10} CFU/g). (Figures 3-2 to 3-6)

3.3.2 Curve Verification

The MPN technique is used to measure the number of microorganisms in food. The MPN values are obtained from MLG 2.05 MPN tables (U.S. Department of Agriculture, 2014), and the microbial population is calculated from the formula used for this technique. The SalQuant® estimates for boot covers, ropes, and spleen and pig feces were verified against MPN estimates. When comparing MPN and SalQuant® for pig feces, boot cover, and ropes samples, there was no statistical difference for the MPN and SalQuant® methods ($P>0.05$), with the exception of spleens ($P=0.0246$).

A method was considered accurate if the mean quantification value was within $\pm$ 0.5 $\log_{10}$ CFU/mL (g) of the inoculated concentration. The MPN method underestimated for 2.0 $\log_{10}$ CFU/mL and 4.0 $\log_{10}$ CFU/g for boot covers and spleen samples, respectively, The MPN method overestimated at 3.0 Log_{10} CUF/g and 2.0 Log_{10} CFU/mL for pig feces and ropes samples, respectively. SalQuant® overestimated at 1.0 Log_{10} CUF/g for spleen samples and 0.0 Log_{10} CUF/g for pig feces samples, and underestimated pig feces samples at 7.0 Log_{10} CUF/g.

For spleen samples, MPN and SalQuant® varied statistically (P=0.0246), with SalQuant® overestimating the 4 log_{10} CFU/g spleen sample.

3.4 Discussion

The *Salmonella* enumeration estimates generated by SalQuant® and MPN from matched samples suggest that SalQuant® can be effectively used in place of the MPN method. The one exception is for spleen samples, for which a significant difference (P=0.0264) was detected between the two methods. While the curve generated by SalQuant® generally meets the statistical requirements (a log RMSE of 0.62 does not fully satisfy the <0.60 previously established threshold), the lack of agreement with the MPN method should be considered for future studies and SalQuant® curve development efforts. It is possible that the dark red color of homogenized spleens creates a matrix interference that impacts SalQuant® efficacy, perhaps by interfering with fluorescence detection.

Applegate et al. (2023) conducted a variety of experiments to demonstrate that SalQuant® is an effective and affordable method for quantifying *Salmonella*. Applegate et al. developed and validated real-time PCR for quantification of *Salmonella* within poultry boot covers, processing rinsates, ground chicken, and ground turkey samples. The study used statistical parameters estimated by linear fit equations, including R^2 values ranging from 0.87 to 0.94, Log RMSE values ranging from 0.35 to 0.44, and a Log_{10} CFU/ mL (g) enumerable range from 0.00 to 4.00. The SalQuant® method was tested against the modified MPN method, and no significant differences were found at any inoculation level (P>0.05). Same as the current project, the SalQuant® and MPN method overestimated or underestimated *Salmonella* levels at some inoculation levels. The MPN method underestimated *Salmonella* levels in the 0.0 and 2.0 Log_{10} CFU/mL (g) range for ground chicken 1:4 and in the 0.0 and 3.0 Log_{10} CFU/mL (g) for ground

chicken 1:6. Boot cover MPN and ground turkey 1:4 MPN overestimated *Salmonella* by 1.00 Log_{10} MPN/g at 3.00 Log_{10} CFU/g, and 2 Log_{10} MPN/g at the 2 Log_{10} CFU/g level, respectively. Additionally, Applegate noted that SalQuant® effectively and accurately estimated the logarithmic level of pre-enriched *Salmonella* in poultry rinse samples within 8 hours compared to Petrifilm™ and MPN. (Applegate et al., 2023).

In 2023, Vargas et al. compared SalQuant® and MPN methods to confirm that BAX® System SalQuant® is capable of accurately enumerating *Salmonella* in pork and beef lymph nodes. Pork and beef lymph nodes were processed and spiked with 0.0 to 5.0 Log_{10} CUF/g of *Salmonella* Typhimurium. The results were statistically analyzed using linear fit equations with a lymph node recovery time of 6 hours reported. When comparing SalQuant® and MPN, there was no significant difference between the two methods (Vargas et al., 2023), which is similar to the results obtained in the present study for all sample types except for spleens.

In 2013, Berghaus et al. conducted a prospective cohort study in collaboration with a commercial broiler production company in northern Georgia using the MPN method for the enumeration of *Salmonella* in broiler houses samples (boot sock, drag swab, fecal samples, litter samples) and processing plant carcass rinses samples (outside plant, rehang, pre-chill, and post-chill) (Berghaus et al., 2013). The highest levels of *Salmonella* were found in boot sock for the farm and outside plant carcass rinses samples, which were 2.34 Log_{10} MPN and 3.44 Log_{10} MPN, respectively (Berghaus et al., 2013). Berghaus noted that the effective methods of pathogen enumeration are necessary to establish and improve the pathogen control in commercial production facilities (Berghaus et al., 2013). The SalQuant® is a reliable, efficient method for enumerating *Salmonella* in a variety of matrices, it can provide faster results

compared to the MPN methods (Applegate et al., 2023). The results of this experiment show that SalQuant® can serve as a valuable tool for food safety and quality control in the pork industry.

3.5 Conclusions

This study developed and validated quantitative PCR BAX® System SalQuant® methods for rapidly enumerating pre-harvest and harvest pig samples. Compared to the MPN method that requires an overnight incubation, SalQuant® can accurately estimate *Salmonella* concentrations within 0 to 10 hours for boot covers, ropes, and pig feces. Additional research is necessary to validate an effective SalQuant® curve for pig spleen *Salmonella* enumeration. The SalQuant® method can be used as a tool for contamination risk assessment in the pork industry and supports the use of real-time PCR-based quantitative methods as a food safety management tool for continuous monitoring of pathogen contamination and risk-based product disposal.

3.6 Figures and Tables

Figure 3-1. SalQuant® Curve Development Experimental Design for Pig Feces (Low and High Level), Spleen, Rope, and Boot Cover Samples

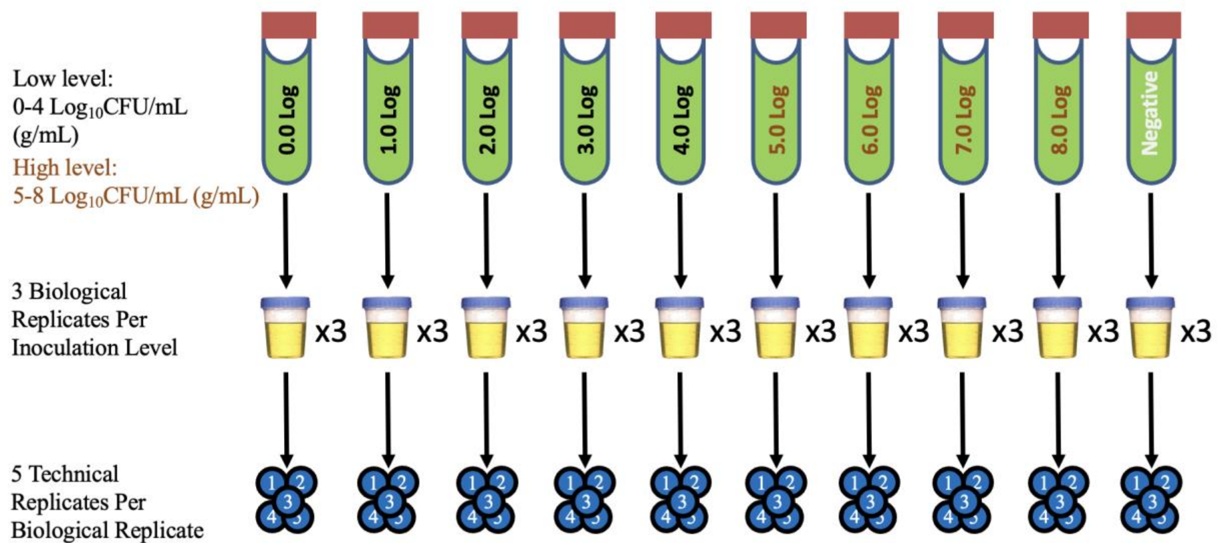

Figure 3-2. SalQuant® Curve Estimations of *Salmonella* Typhimurium 14028 in Log_{10} CFU/mL of Boot Covers Collected from a Market Pig Barn According to Inoculation Concentration (Log_{10} CFU/mL).

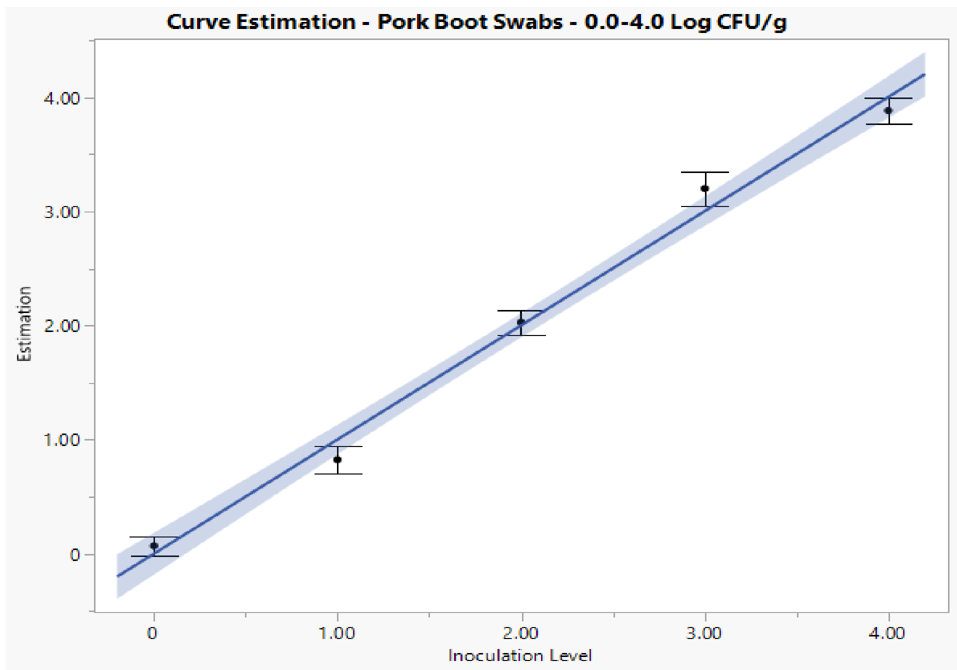

Figure 3-3. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Spleens Collected from a Large Commercial USDA-FSIS Inspected Pork Producer According to Inoculation Concentration (Log_{10} CFU/mL).

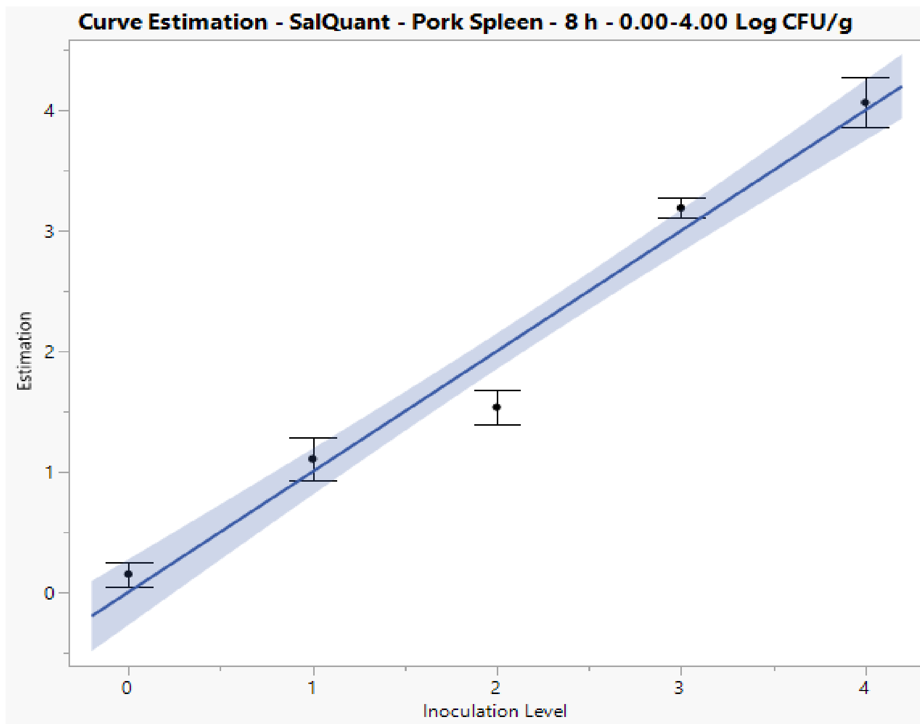

Figure 3-4. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Ropes Collected from a Large Commercial USDA-FSIS Inspected Pork Producer According to Inoculation Concentration (Log_{10} CFU/mL).

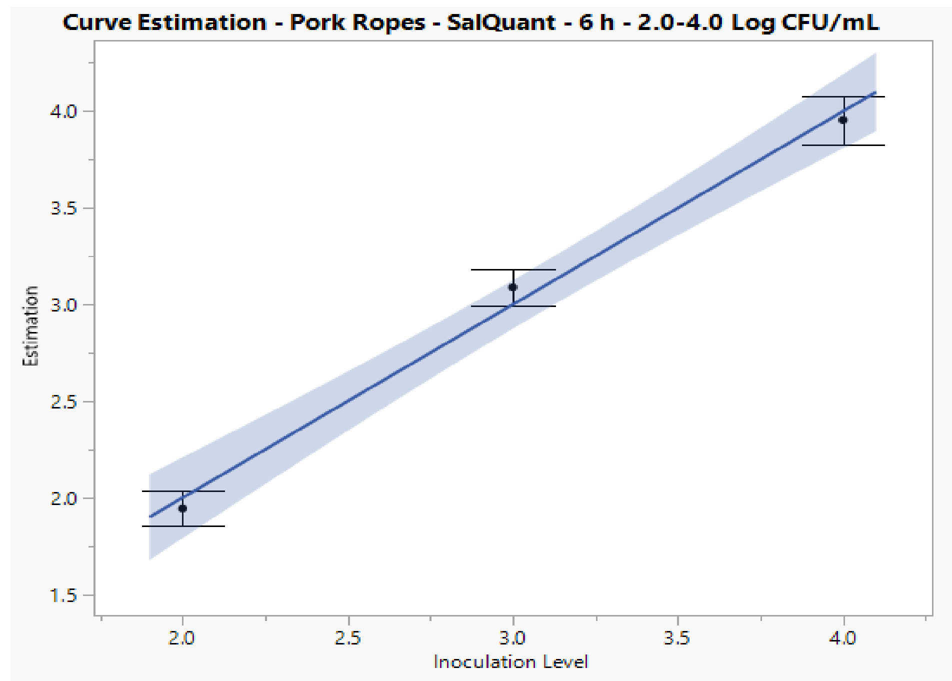

Figure 3-5 SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Pig Feces Collected from a Market Pig Barn by Inoculation Level 0-4 Log_{10} CFU/mL

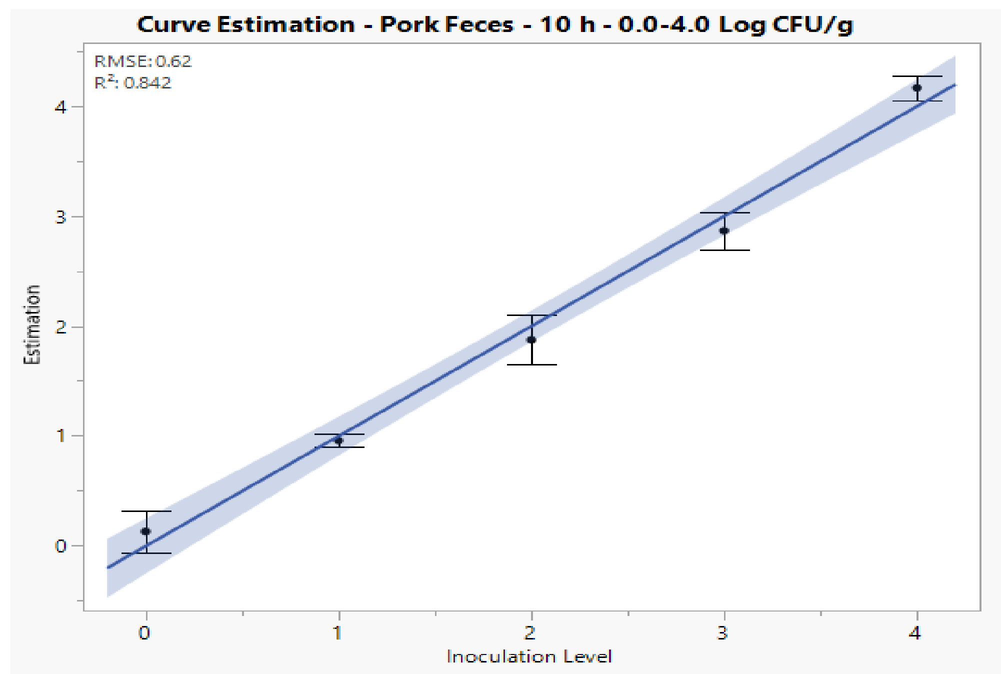

Figure 3-6. SalQuant® Curve Estimations of *Salmonella* Typhimurium 10428 in Log_{10} CFU/mL(g) of Pig Feces Collected from a Market Pig Barn by Inoculation Level 5-8 Log_{10} CFU/mL

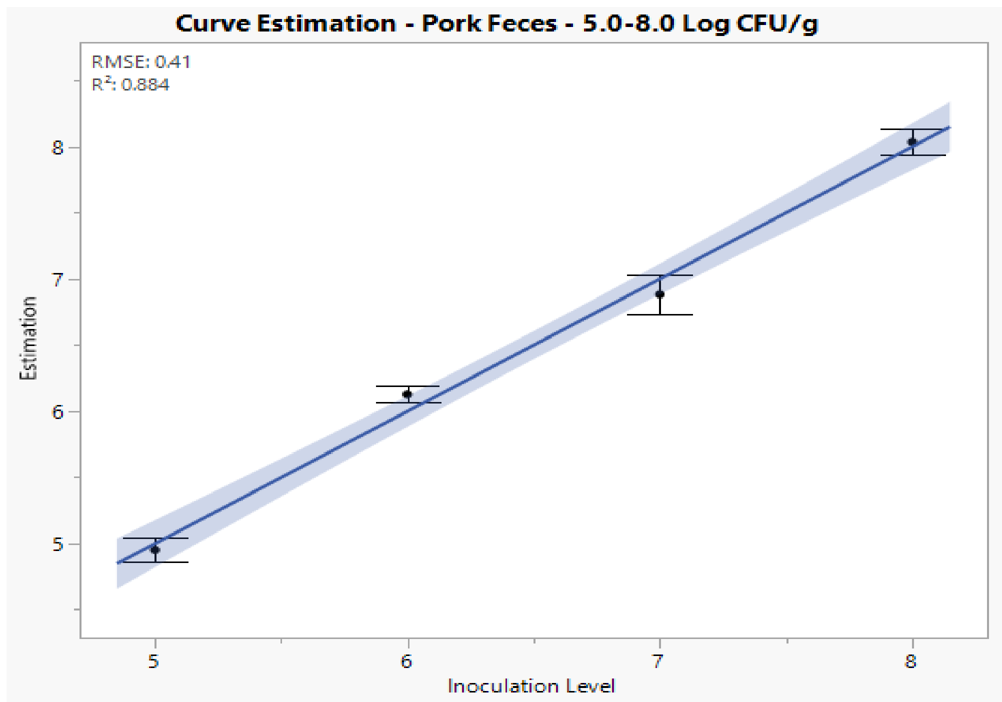

Table 3-1. BAX® System SalQuant® Curve Development of *Salmonella* Typhimurium 10428 Parameters of Boot Cover, Ropes, Spleen, and Pig Feces Matrices.

Matrix	**Timepoint (h)**	**R^2**	**Log RMSE***	**Enumerable Range**
Boot Cover	8	0.91	0.46	0.00 - 4.00 Log_{10} CFU/mL
Ropes	6	0.81	0.40	2.00 - 4.00 Log_{10} CFU/mL
Spleen	8	0.83	0.62	0.00 - 4.00 Log_{10} CFU/g
Pig Feces	10	0.84	0.61	0.00 - 4.00 Log_{10} CFU/g
Pig Feces	0	0.88	0.41	5.00 - 8.00 Log_{10} CFU/g

*Root Mean Square Error

Table 3-2. BAX® System SalQuant® Linear-Fit Curve Estimations of *Salmonella* Typhimurium 10428 Compared to MPN Estimations for Matched Biological Replication Samples.

Matrix	Inoculation Level*	SalQuant® Estimation*	MPN[s] Estimation*	*P* Value[γ]
Boot Covers	0.0	0.1	-0.1	0.9208
	1.0	0.8	1.0	
	2.0	1.8	**1.4**	
	3.0	3.0	3.3	
	4.0	3.9	4.0	
Ropes	2.0	2.2	**2.7**	0.0874
	3.0	3.0	3.2	
	4.0	3.8	4.0	
Spleens	0.0	0.0	-0.4	0.0246
	1.0	**1.8**	1.0	
	2.0	2.1	1.7	
	3.0	2.9	2.7	
	4.0	4.5	**3.2**	
Pig Feces	0.0	**0.6**	0.2	0.3924 low 0.5920 high
	1.0	1.0	1.3	
	2.0	1.7	2.4	
	3.0	3.1	**3.7**	
	4.0	4.2	4.0	
	5.0	5.0	5.3	
	6.0	6.2	6.4	
	7.0	**6.4**	6.7	
	8.0	8.1	7.7	

*Log CFU/mL (g)

[s]MPN/mL (g)

[γ] α=0.05

Bolded Values: Indicates mean quantification value exceeds +/- 0.5 Log CFU/mL allowable margin from target concentration.

References

Applegate, S. F., Englishbey, A. K., Stephens, T. P., & Sanchez-Plata, M. X. (2023). Development and verification of a poultry management tool to quantify *Salmonella* from live to final product utilizing RT-PCR. *Foods*, *12*(2), 419. https://doi.org/10.3390/foods12020419

Bari, M. L., & Yeasmin, S. (2022). Microbes culture methods. In N. Rezaei (Ed.), *Encyclopedia of Infection and Immunity* (pp. 77-98). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-818731-9.00128-2

Berghaus, R. D., Thayer, S. G., Law, B. F., Mild, R. M., Hofacre, C. L., & Singer, R. S. (2013). Enumeration of *Salmonella* and *Campylobacter* spp. in Environmental Farm Samples and Processing Plant Carcass Rinses from Commercial Broiler Chicken Flocks. *Applied and Environmental Microbiology*, *79*(13), 4106-4114. https://doi.org/doi:10.1128/AEM.00836-13

Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., & Pasmans, F. (2008). Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and control. *Veterinary Microbiology*, *130*(1-2), 1-19. https://doi.org/10.1016/j.vetmic.2007.12.017

Centers for Disease Control and Prevention. (2019). *Questions and answers*. Retrieved March 20, 2023 from https://www.cdc.gov/salmonella/general/index.html#print

Dickson, J. S., Dept. of Microbiology, C. o. A., University;, I. S., H.S. Hurd & M. H. Rostagno, Unit;, P.-H. F. S. a. E. D., National Animal Disease Center, Agricultural Research

Service, USDA, & Iowa, A. (2019). Salmonella *in the Pork Production Chain*. Retrieved May 23, 2023 from https://swine.extension.org/salmonella-in-the-pork-production-chain/

Food Safety and Inspection Service. (2022). *Changes to the* Salmonella *verification testing program: proposed performance standards for* Salmonella *in raw comminuted pork and intact or non-Intact pork cuts and related agency verification procedures*. Retrieved May 24, 2023 from https://www.federalregister.gov/documents/2022/02/16/2022-03301/changes-to-the-salmonella-verification-testing-program-proposed-performance-standards-for-salmonella

Hoy, M. A. (2013). Chapter 8 - DNA amplification by the polymerase chain reaction:molecular biology made accessible. In M. A. Hoy (Ed.), *Insect molecular genetics (Third Edition)* (pp. 307-372). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-415874-0.00008-1

Kahya, S., & Demirbilek. (2017). Salmonellosis in Animals. In M. Maria Teresa (Ed.), *Salmonella* (pp. Ch. 2). IntechOpen. https://doi.org/10.5772/intechopen.72192

Lopez, R. B. (2022). *Development and implementation of standardized microbial quantification-based procedures for process control in pork operations* Texas Tech University].

Matthews, K., Kniel, K. E., & Montville, T. J. (2017). 14. *Salmonella* Species. In *Food Microbiology - An Introduction (4th Edition)* (pp. 246). American Society for Microbiology (ASM). https://app.knovel.com/hotlink/pdf/id:kt011EDR31/food-microbiology-an/salmonella-species

U.S. Department of Agriculture. (2014). *MLG Appendix 2.05. Most Probable Number Procedure and Tables.* U.S. Department of Agriculture Retrieved from https://www.fsis.usda.gov/sites/default/files/media_file/2021-03/MLG-Appendix-2.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. https://www.mdpi.com/2304-8158/12/4/822#

Chapter 4 - Exploring the Use of Probicon L28 and BIOPLUS® 2B as Direct-Fed Microbials to Reduce *Salmonella* and Shiga Toxin-Producing *Escherichia coli* in Market Pigs

Abstract

Pigs are hosts for *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) and these pathogens can commonly be isolated from the pig farm environment. Pigs can carry pathogens to the abattoir and contaminate pork products, posing a risk to public health. Identifying an intervention that effectively reduces pathogens in commercial pigs before harvest is imperative. Due to the need for effective pre-harvest interventions in the pig industry, the objective of this study was to investigate BIOPLUS® 2B (*Bacillus licheniformis* and *Bacillus subtilis*) and Probicon L28 (*Lactobacillus salivarius* L28) as pre-harvest interventions to reduce *Salmonella* and STEC in commercial growing-finishing pigs. Two groups of pigs (group 1 N=294; group 2 N=356, initial BW = 116.2 lb) were fed a standard corn-SBM finishing diet according to the following treatments: Probicon L28 supplementation through water lines at 1.0×10^6 CFU/head/day (Probicon); BIOPLUS® 2B supplemented at 3.0×10^9 CFU/head/day (BIOPLUS® 2B); and a control with no added probiotic (Control). With each group of pigs, 12 pens were used per treatment (n=24 total), for a total of 36 pens per group (n=72 pens total). Each group was sampled upon arrival/baseline, mid-way through the grow-finish phase/6wk post-placement, and prior to loadout/13wk post-placement to collect fecal samples (4 pigs/pen), boot covers (2/pen), and ropes (1/pen). Market pigs were followed to the abattoir and superficial inguinal lymph nodes (SILNs) were collected. *Salmonella* and Shiga toxin-producing *Escherichia coli* were detected in all samples using the BAX® System. Overall *Salmonella* prevalence was very

low for all sample types at less than 3%, and *Escherichia coli* O157:H7 was not detected in any samples throughout the study. When compared to the control, there was no evidence (P>0.05) that BIOPLUS® 2B and Probicon L28 impacted the prevalence of STEC (*stx* and *eae* genes) or serogroups O26, O121, O45, O103 and O145 in feces, boot covers, ropes, and SILNs of market pigs.

4.1 Introduction

According to Centers for Disease Control and Prevention estimates, foodborne diseases cause 48 million people to become ill, 128,000 people to be hospitalized, and 3,000 people to die each year (Centers for Disease Control and Prevention, 2022). *Salmonella*, Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria monocytogenes* are considered major causes of foodborne disease outbreaks in the United States (Marshall et al., 2020). Non-typhoidal *Salmonella* is responsible for the most hospitalizations and deaths from foodborne illnesses (Centers for Disease Control and Prevention, 2022). An estimated 265,000 STEC infections occur annually in the United States, of which 36% are caused by STEC O157 and 64% by non-O157 STEC (Centers for Disease Control and Prevention, 2014). While the CDC can present estimates, the real count of infections is probably to be much higher because not all STEC infections are diagnosed, either because many infected people do not seek health care or because many patients who do seek health care do not provide stool samples for testing (Centers for Disease Control and Prevention, 2014).

According to Dr. Steven Larsen, Assistant Vice President of Science and Technology, National Pork Board, “Safe pork starts with healthy pigs” (Larsen, n.d.). For the pork industry, *Salmonella* is the major foodborne pathogen associated with pork, and pork can become contaminated at any stage of production or throughout the supply chain (Larsen, n.d.). Studies

have reported that STEC is present in pork products, although often at much lower levels than in ruminant products, and producers and consumers still need to be concerned about possible STEC contamination in pork products (Svboda & Cutter, 2012). Similar to *Salmonella*, STEC contamination originates at the farm and can be transferred from the pig to the carcass during slaughter, thereby contaminating pork and commodities (Colello et al., 2016).

Conversely, *Salmonella* and STEC not only cause a health threat to humans but can also make pigs sick and lead to economic losses. Infection with *Salmonella* can present in pigs in two ways, clinical or subclinical (Pipestone Veterinary Services, 2018). Clinical signs most commonly present as diarrhea or sepsis, whereas the majority of pig herds present as subclinical *Salmonella* that is a more common presentation in pig farms (Pipestone Veterinary Services, 2018). *Salmonella* causes economic losses in the pig industry, with salmonellosis resulting in increased drug use, reduced weight gain and feed to gain ratio, prolonged time to market, and loss of productivity due to changes in carcass weight (Ainslie-Garcia et al., 2018). In pigs, STEC of the Stx2e subtype cause edema disease (Remfry et al., 2021), which mainly affects healthy, rapidly growing nursery pigs, with varied clinical signs that may include marked edema of the gastric submucosa and mesocolon, sudden death in pigs, and paralysis (Fairbrother, 2023). Edema disease caused by Stx2e-producing *E. coli* can cause serious economic challenges on pig farms, with the older the pigs the greater the economic losses (Mesonero-Escuredo et al., 2021).

Probiotics are generally considered safe and are often recognized for the many benefits they confer to humans and animals, including protection against pathogen infection and regulation of gut microbiota (Shi et al., 2020). Probiotics containing *Lactobacillus* are effective at reducing foodborne pathogens in feces and hides, and their potential to replace antibiotics has increased their use in the livestock industry (Ayala et al., 2017). Probiotics have also been reported to

improve the intestinal barrier by producing antibacterial compounds such as bacteriocins and organic acids, competing with pathogenic bacteria for epithelial receptors and nutrients in the gastrointestinal tract, and producing enzymes and vitamins (Ayala et al., 2017). *Lactobacillus salivarius* L28 (Probicon) is a newly identified type of *Lactobacillus* that was isolated from ground beef (Flach et al., 2022). Preliminary data suggest that *Lactobacillus salivarius* L28 control *E. coli* and *Salmonella* in cattle feces, and has similar effects to subtherapeutic antibiotics in terms of weight gain, performance, and carcass traits in beef cattle, and it can be considered as an alternative to reduce the cattle industry's dependence on antibiotics (Flach et al., 2022). *Bacillus subtilis* and *Bacillus licheniformis* are considered probiotics because of their ability to produce enzymes and secondary metabolites that inhibit the growth of pathogenic microorganisms (Romo-Barrera et al., 2021). The combination of *Bacillus licheniformis* and *Bacillus subtilis* has been shown to have anti-inflammatory and antioxidant effects, which inhibit the pathogenic bacteria adhesion (Palkovicsné Pézsa et al., 2022). BIOPLUS® 2B is a probiotic product, consisting of *Bacillus licheniformis* and *Bacillus subtilis*, and is commonly used in the pig industry (Chr Hansen, 2023). The objective of this study was to evaluate the supplementation of BIOPLUS® 2B and Probicon L28 as a pre-harvest intervention to reduce *Salmonella* and Shiga toxin-producing *Escherichia coli* in market pigs.

4.2 Materials and methods

4.2.1 Experimental Design

The study was conducted at the Kansas State University Swine Research and Teaching Facility and the protocol for this experiment was approved by the Kansas State University Institutional Animal Care and Use Committee (#4485). Each pen was fitted with a 2-hole dry single sided feeder (Farmweld Teutopolis, IL) and a 1-cup waterer to provide feed and water.

Each pig had 0.72 square meters of floor space and was located on a fully slatted concrete floor with a 1.21-meter pit underneath for liquid storage. Pens were organized by treatment in the barn (Figure 4-1 and 4-2) and barriers made of hard plastic dividers were erected between pens of different treatments to eliminate nose to nose contact between animals of different treatments. Only pens of the same treatment were allowed nose to nose contact between pens. Two groups of market pigs were included in this project (N=294 pigs in the first group; Group 2 N=356, initial BW = 116.2 lb). A total of 36 pens were used for each group of commercial pigs, 12 pens were used for each treatment, with 8-10 pigs per pen. Each pen was assigned to one of three treatment diets.

Each pen was sampled at each of three different occasions throughout the feeding period: 1) upon arrival (baseline measurement), 2) midway (approximately 6 weeks on trial), and 3) before loadout (approximately 13 weeks on trial). The feces of four pigs were randomly collected, two boot covers from the person who collected feces samples in each pen, and a rope that was hung on each pen allowed pigs to chew during each sampling period. On the last day of the trial, the final pen weight and individual weight of the pigs were obtained, and the pigs were tattooed with unique identification by pen to facilitate identification of each treatment, then shipped to a USDA-FSIS inspected processing facility for collection of superficial inguinal lymph nodes, MicroTally™, and trim samples. During shipment, the pigs were loaded onto a truck according to treatments, and one treatment was placed on each truck. In lairage, the pigs were maintained as separate treatments, with 1-2 lairage pens per treatment. All samples were analyzed for STEC (*stx, eae* genes and, O157:H7, and O26, O111, O121 O45, O103, O145 serogroups) and *Salmonella* using the BAX® System. Immunomagnetic separation (IMS) was used to isolate *Salmonella* and STEC for further characterization.

4.2.2 Treatment Diets

The pen was considered the experimental unit and was assigned to one of three treatments: 1) a standard corn-SBM finishing diet with Probicon L28 supplemented through water lines with a target concentration of 1.0×10^6 CFU/head/day using a water medicator system (Model D14MZ10; Dosatron International, Clearwater, FL), which diluted the stock solution to water ratio approximately 1:100 to achieve a target concentration of 1.0×10^6 CFU/ head/day, 2) a standard corn-SBM finishing diet supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of feed; ~3.0×10^9 CFU/head/day), and 3) the control group was fed a standard corn-SBM diet. Diets were fed in 3 phases and all diets were manufactured at a commercial feed mill (Hubbard Feeds; Beloit, KS). A robot was used as a feeding system (FeedPro; Feedlogic Corp, Wilmar, MN) and recorded the amount of feed provided for this project.

The Probicon L28 stock solution was prepared daily and consisted of tap water, activated Probicon L28 and electrolyte powder, with pH of the stock solution adjusted to 6.5. Briefly, 2 g of Probicon Calf (NexGen Innovations, LLC, Lubbock, TX) were combined in 10 mL of tryptic soy broth (TSB; BD BioSciences; Franklin Lakes, NJ) and activated for 10 hours at 37°C and stored at 4°C until use (up to 72 hours). Each day, a fresh Probicon L28 stock solution was prepared in a clean 5-gallon bucket by combining 17 kg of tap water with 17 g of electrolyte powder (DuMor Multi-Species Electrolytes Supplement, distributed by Tractor Supply Company, Brentwood, TN) and one tube of activated Probicon L28. Prior to adding the Probicon L28, the stock solution pH was adjusted to ~6.5 using distilled white vinegar (Great Value, Bentonville, AR). The pH of each stock solution was confirmed by pH strips (two brands were used due to supply chain challenges: API, McLean, Virginia; Dr. Tim. Wang, Amazon). The average estimated Probicon intake of Probicon treatment pigs was calculated based on 1) the

daily pig water intake record, and 2) laboratory estimates for stock solution concentration, with group 1 consuming an average of 6.7×10^{4} CFUs per pig per day and group 2 consuming 1.2×10^{5} CFUs per pig per day.

4.2.3 Pre-Harvest Sample Collection

Pig Feces

Feces were collected from four pigs at random in each pen. Using a gloved hand, one finger was inserted into the anus and gently rotated in a circular motion to manually stimulate defecation. Gloves were changed after each fecal sample was collected to ensure no cross contamination. Each sample was placed into a 24 oz Whirl-Pak bag (Nasco, Fort Atkinson, WI), and bags were stored in a cooler with ice packs for further processing.

Boot Covers

During fecal sample collection, one person entered the pen wearing a double layer of boot covers, where the internal boot cover provided a barrier between the external boot cover and the boot. Once the pig feces were collected, the outer layer boot covers were placed into separate 55 oz Whirl-Pak bags (2 boot cover samples per pen), and the inner layer boot covers were discarded. Gloves were replaced after each boot cover sample collection to ensure no cross contamination. Sample bags were stored in a cooler with ice packs for further processing.

Ropes

Twisted cotton blend ½-inch ropes (Koch, Tractor Supply Company, Manhattan, KS) were cut to a 24-inch length and tied to the fence of each pen to ensure that pigs could chew the rope. Rope samples were collected at each sampling point and placed into a 55oz Whirl-Pak bag after thirty minutes. Gloves were changed after each rope collection to ensure no cross contamination. After collection, each sample was placed in a cooler with ice packs until further processing.

4.2.4 Harvest Sample Collection

Lymph nodes

Market pigs were shipped on separate trucks (one truck per treatment) to a large commercial USDA-FSIS-inspected pork producer and superficial inguinal lymph nodes (SILNs) were collected. The SILNs (group 1 N=262; Group 2 N= 314) were placed in zip top bags and SILNs from different treatments were placed in separate bags. After collection, each sample bag was placed in a cooler with ice packs until further processing.

Trim

When the head was split, head and cheek meat trim from each hog was collected and loaded into plastic totes, with a separate tote for each treatment. Each treatment of trim samples was randomly sampled using clean forceps, and enough trim was collected to fill one 55oz. Whirl-Pak homogenizer blender filter bags. All sample bags were placed in a cooler with ice packs until further processing.

MicroTally™

Head and cheek meat trim from each diet was held in plastic totes, with a separate tote for each treatment. Using a MicroTally™ cloth (Fremonta; San Jose, CA), each tote was manually sampled for 30 seconds using one side of the cloth, followed by another 30 seconds using the second side of the cloth. Gloves were changed between each treatment. After collection, MicroTally™ cloths were returned to their original plastic sample bag and stored in a cooler with packs until further processing.

Ropes in Lairage

At the abattoir, the pigs were divided into lairage pens according to their treatment. Ropes with 3 to 4 inches length were hung by the processor in each lairage pen and provided for each

treatment. After collection, rope samples were stored in a cooler with ice packs until further processing.

4.2.5 Pre-Harvest Sample Processing

Pig Feces

Pig fecal samples (N=144 per sampling period) were weighed to ensure a 10 g sample was mixed with 90 mL pre-warmed (42˚C) BAX MP media (Hygiena, Camarillo, CA) containing Quant solution (QS: Hygiena, Camarillo, CA) at a concentration of 0.5 mL/L to prepare a feces homogenate (FH). Ten mL of 42˚C pre-warmed BAX MP media with QS at a concentration of 0.5 mL/L was mixed with 10 mL FH into sterile Whirl-Pak bags to make 20 mL of diluted FH samples (dFH). All 20 mL dFHs were incubated at 42˚C for 24 hours.

Boot Covers

Boot cover samples (N=72 per sampling period) were stomached by a Smasher (bioMérieux, Marcy-l'Étoile, France) with 100 mL of buffered peptone water (BPW;Hygiena, Camarillo, CA) and homogenized for 1 minute to prepare a boot cover homogenate (BCH). Thirty mL of BCH was transferred into a Whirl-Pak bag and mixed with thirty mL pre-warmed (42˚C) BAX MP containing 1 mL/L QS as diluted BCH (dBCH). All dBCHs were incubated at 42˚C for 24 hours.

Ropes

Rope samples were hand massaged for 1 minute with 300 mL of prewarmed BAX MP with QS at a concentration of 1 mL/L. Rope homogenate (RH) was incubated at 42˚C for 24 hours.

4.2.6 Harvest Sample Collection

Lymph nodes

Superficial inguinal lymph nodes were trimmed to remove all surrounding fat and fascia, weighed and then boiled for 3- 5 seconds. The SILNs were placed into Whirl-Pak filter bags (24 oz) and smashed by a rubber mallet. Twenty mL pre-warmed (42˚C) BAX MP media was added to SILNs of 0 to 3 grams and 80 mL pre-warmed (42˚C) BAX MP was added to SILNs greater than 3 grams and homogenized by a smasher for 1 minute, resulting ina lymph node homogenate (LNH). All LNH samples were incubated at 42˚C for 24 hours.

Trim

Head and cheek meat samples were processed using the N60 method, where 60 small pieces totaling 375 g were homogenized with 1,500 mL of pre-warmed (42˚C) BAX MP. All trim homogenate (TH) samples were incubated at 42˚C for 24 hours.

MicroTally™

Each MicroTally™ was weighed, homogenized by a smasher with 200 mL pre-warmed (42˚C) BAX MP for 1 minute, and incubated at 42˚C for 24 hours.

Ropes

Rope samples were added to 300 mL of prewarmed BAX MP with QS at a concentration of 1 mL/L and homogenized by hand for 1 minute. Rope homogenate (RH) was incubated at 42˚C for 24 hours.

4.2.7 STEC and Salmonella *Detection*

After incubation, the BAX® System Real-Time STEC Screening Suite [STEC screen (*stx, eae* genes), Panel 1 (O26, O111, O121 serogroups), Panel 2 (O45, O103, O145 serogroups), *E. coli* O157:H7 EXACT] and BAX® System Real-Time *Salmonella* Assay were utilized to detect STEC and *Salmonella* for all samples. Immunomagnetic separation (IMS) was used for all pig

feces, boot cover, ropes, and SILN samples positive for *Salmonella* based on BAX® System results. The IMS methods are described below in section 4.2.9.

4.2.8 STEC and Salmonella *Isolation and Characterization*

STEC

Immunomagnetic separation was used for STEC positive SILN samples based on BAX® System results. Automatic IMS (KingFisher™; Thermo Scientific™, Waltham, MA) was conducted according to manufacturer's recommendations using IMS bead (Romer Lab, Newark, Delaware) pools that matched the O serogroup results provided by the BAX® System. Following IMS, 5 uL was removed from the IMS tube for 1:50 dilution by PBS-Tween (PBS-T; Thermo Fisher; Waltham, MA), 50 µL diluted beads were spread plated on ChromAgar STEC (ChromAgar, Paris, France), 50 µL undiluted beads were plated on ChromAgar STEC, and a 10 µL loop was used to streak undiluted beads on ChromAgar STEC. If samples were positive for STEC (*stx and eae*) but not an O group based on BAX® System results, they were streaked to ChromAgar STEC directly from the sample. All plates were incubated at 37˚C for 24 hours. Pink colonies were picked, with at least four colonies on each plate, and two additional colonies were picked when using more than one O group bead in a pool for IMS. All pink colonies were transferred to 96-well blocks filled with 1 ml TSA (BD Biosciences; Franklin Lakes, NJ) in each well and incubated at 37˚C for 24 hours. The 96-well blocks were refrigerated and sent to the United States Meat Animal Research Center (USMARC) for serotyping and further characterization.

Salmonella

The lymph node, trim, MicroTally™, and rope samples collected during harvest that were identified as positive using the BAX® System were streaked on xylose lysine desoxycholate

(XLD) agar (Remel, Thermo Fisher, Waltham, MA). Automatic IMS was conducted using manufacturer's recommendations for all pig feces, boot cover, and rope samples during pre-harvest sample collections. When direct streaking was unable to recovery a colony for lymph node, trim, MicroTally^{TM}, and rope samples collected during harvest stage were subjected to automatic IMS. One hundred microliter of bead-bacteria complex generated by IMS were transferred to 3 mL Rappaport Vassiliadis (RV; BD BioSciences; Franklin Lakes, NJ) broth, and incubated at 42˚C for 18 hours. Incubated RV tubes were streaked for isolation on XLD agar, and all XLD plates were incubated at 37˚C for 24 hours. After incubation, three well-isolated, presumptive *Salmonella* colonies (black on XLD) from each plate were re-streaked on XLD (one plate per colony), and incubated 37˚C for 24 hours. One well-isolated colony from each re-streak plate was picked and transferred to a 10 mL tube of TSB, and TSB tubes were incubated 37˚C for 24 hours. Each TSB tube was confirmed as *Salmonella* using the BAX® System Real-Time *Salmonella* Assay. One mL from each TSB tube that confirmed as *Salmonella* on the BAX® System was frozen with 10% glycerol (BD BioSciences; Franklin Lakes, NJ) in microtubes in triplicate at -80°C.

Frozen isolates were removed from the -80°C freezer, streaked for isolation onto XLD agar and incubated at 37˚C for 24 hours. Individual black colonies were transferred to 96-well blocks filled with 1 mL TSA in each well and incubated at 37˚C for 24 hours. The culture was refrigerated and sent to USMARC for serotyping.

4.2.9 STEC and Salmonella *Serotyping*

Salmonella

A sample of 1 μL loop was suspended into 1000 μL distilled water and heated to 95°C for 2 min (Kim et al., 2006). The cells were cleaved to be used as DNA templates (Kim et al., 2006).

Samples were analyzed by the multiplex PCR (Herrera-León et al., 2007). Five pmol of primer, 1.5 mM $MgCl_2$, and 5 milliliters suspension were used for multiplex PCR reaction (Herrera-León et al., 2007). The fragments underwent separation through unidirectional electrophoresis in a 2.5% agarose gel, utilizing TAE×1 buffer, and were subsequently visualized by ethidium bromide staining (Herrera-León et al., 2007). Fifty and 100 bp DNA ladders were used for fragment sizes determination (Herrera-León et al., 2007).

STEC

PCR Master Mix 500 rxns were used for determining O serogroups of O26, 45, 103, 111, 121 and 145. The PCR Mix was combined 1375 μL 10× Buffer II with 15 mM $MgCl_2$, 510 μL of O-Group I Primer Mix, 250 μL dNTPs, and 9.75 mL ddH_2O. The number of samples were determined, 19.6 μL of the PCR Mix was mixed with 0.4 μL of Taq polymerase for each sample. The EHEC-59 PCR program was run with a series of temperature cycles. After PCR, the resulting products were analyzed on a 1.7% agarose gel through electrophoresis, and the O serogroups are interpreted based on the specific product sizes.

4.2.10 Statistical Analysis

Statistical analysis was conducted by Dr. Qing Kang of the Kansas State University statistical consulting laboratory as described. Statistical analyses were performed separately for each sample type and prevalence type (STEC and individual serogroups). 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).

For feces and boot cover samples, prevalence data were organized at the pen level as a binomial outcome. The binomial prevalence data at 6 and 12 weeks were analyzed using the

logit linear mixed model. Fixed effects of the model included Rep, treatment, time and treatment-by-time interaction. The pen-level prevalence rate at baseline served as a numeric covariate. The random effect was pen. 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 model-based estimates of prevalence rates, their 95% Wald confidence intervals, and odds ratios. Statistical analyses were carried out using the SAS GLIMMIX procedure.

For rope samples, the prevalence data at 6 and 12 weeks were analyzed separately, at each sampling time, using the exact conditional logistic regression approach because of limited sample size (i.e. one sample per pen at each sampling time). The baseline prevalence status (consisting of three levels: either negative, positive, or missing) served as a categorical covariate. For SILN samples, the prevalence data were analyzed using the exact conditional logistic regression approach because of low overall STEC prevalence rate (18/576≈3.1%). In both analyses, rep served as the stratifying variable for the conditional inference and the fixed effect was treatment. The estimated prevalence rates of treatments were not available in conditional inference. Therefore, the raw prevalence rates and their 95% Pearson-Clopper confidence intervals of each treatment were reported. Treatments were compared via exact odds ratios and exact score-test P-values. Statistical analyses were performed using the SAS LOGISTIC and FREQ procedures.

4.3 Results

4.3.1 STEC and Serogroup Prevalence

Pig Feces

The main effect of treatment did not significantly ($P>0.05$) impact the prevalence of STEC (*stx* and *eae* genes) or serogroups O26, O121, O45, O103 and O145 in fecal samples of market

pigs at loadout. The main effect of time was significant for STEC, O26, O45, and O145 (P<0.05), with prevalence decreasing throughout the feeding period. A treatment by time interaction was observed for STEC (P=0.001) and O121 (P=0.003), and nearly observed for O26 (P=0.052) (Table 4-1). In comparison to the control, significant differences only occurred at the 6-week sampling point. A statistical difference was observed between BIOPLUS® 2B and the control at 6 weeks, with the prevalence of STEC, O26, and O121 higher (P<0.05) in BIOPLUS® 2B than the control (Table 4-1). Similarly, the O26 and O121 prevalence was higher (P<0.05) at 6 weeks in pigs fed the Probicon diet in comparison to the control diet. By the 13-week sampling point (prior to loadout), STEC, O26, and O121 prevalence in pigs treated with BIOPLUS® 2B and Probicon L28 were not different than the control (P>0.05). The prevalence of O111 was very low throughout the trials, and no fecal samples were positive for *E. coli* O157:H7.

Boot covers

The main effect of treatment did not significantly (P>0.05) impact the prevalence of STEC (*stx* and *eae* genes) or serogroups O26, O121, O45, O103 and O145 in boot covers worn in the pens of market pigs. The main effect of time was significant for STEC and all serogroups (P<0.05), except for O103 (P=0.514). In general, prevalence decreased throughout the feeding period. A treatment by time interaction was not observed for STEC (*stx* and *eae* genes) or serogroups O26, O121, O45, O103 and O145. The prevalence of O111 was very low throughout the trials, and no boot covers were positive for *E. coli* O157:H7. For six pens, boot cover samples collected at 6 and 13 weeks in group 1 and at baseline in group 2 were contaminated and, therefore, excluded from data analysis and statistical modeling.

Ropes

The prevalence of STEC, O26, O121, O45, O103, and O145 serogroups from ropes hung in pens of pigs fed diets supplemented with BIOPLUS® 2B and Probicon L28 did not vary at 6 weeks or 13 weeks (P>0.05) in comparison to ropes from pens of pigs fed the control finishing diet. *Escherichia coli* O157:H7 and O111 were not detected throughout the trial in rope samples. Ten samples at baseline in group 1, 3 samples at 6 weeks and 6 samples at 13 weeks in group 2 were not able to be collected and thus not used for data analysis and statistical modeling.

Lymph Nodes

From the 576 SILNs collected, the BAX® System detected STEC (*stx* and *eae* genes) and serogroups O26, O121, and O145. In comparison to the control, finishing diets supplemented with BIOPLUS® 2B and Probicon L28 did not impact prevalence (P>0.05). Although not significant (P>0.05), a numerical reduction in STEC and O121 prevalence was observed for BIOPLUS® 2B and Probicon L28, in comparison to the control group (Table 4-2).

Trim and MicroTally™

For group 1 pigs, one trim sample collected from the control treatment tested positive of STEC (*stx* and *eae* genes), O26, O121, O45, O103, and O145. One rope from the Probicon treatment was positive for O26, O121 and O103. A rope from the control treatment was positive for O121, O45 and O145. A rope from BIOPLUS® 2B was positive for O121 and O103. One trim sample from the BIOPLUS® 2B treatment of group 2 tested positive for STEC (*stx* and *eae* genes), O26, O121, O45, O103, and O145.

4.3.2 Salmonella *Prevalence*

Salmonella prevalence from was very low throughout this study. Feces collected from group 1 and group 2 had an overall *Salmonella* prevalence of 0.7%, 1.4% 2.8% and 0%, 0%, 0%, at the beginning and end of the feeding period, respectively. *Salmonella* prevalence in boot

covers of group 1 and group 2 market pigs was 2.8%, 1.4%, 0.0% and 0%, 2.8%, 0.0%, respectively. The *Salmonella* prevalence of rope samples from group 1 and group 2 was 0.0%, 2.8%, 0.0% and 0.0%, 0.0%, 10%. *Salmonella* prevalence of SILNs in group 1 and group 2 market pigs was 0.8% and 0%, respectively. All trim (n=6), MicroTally™(n=6), and rope samples (n=6) collected from the abattoir were *Salmonella* positive.

4.3.3 STEC and Salmonella *Isolation*

Of the 18 SILNs, 3 rope, and 2 trim samples positive for STEC, automatic IMS recovered isolates from 7 samples. A total of 150 isolates were recovered, and 57 isolates were characterized as O121 with *stx2e* only (lack *eae*). Of these 57 isolates, 39 were recovered from lymph node samples collected group 1 pigs fed the control treatment. Thirteen isolates were recovered from a lymph node sample and five isolates were recovered from a trim sample collected from group 2 pigs fed the BIOPLUS® 2B diet (Table 4-3).

Among the 2,087 samples included in this study, *Salmonella* was isolated from 28 samples. From each positive sample, up to three colonies were isolated and subjected to serotyping for a total of 78 *Salmonella* isolates. From these 78 isolates, 23 were identified as *S.* Agona that originated from boot covers (6) and feces (3) MicroTally™ (4), ropes (8), and trim (2). Twelve were identified as *S*. Senftenberg that isolated from pig feces (3), boot covers (3), and ropes (6). Nine were identified as *S.* Braenderup, in which isolate from pig feces (3), boot covers (3), and ropes (3). Nine were identified as *S.*monophasic Typhimurium that isolated from trim (3), rope (1), and MicroTally™ (5). Six *S*. Worthington were isolated from boot cover (6). Five *S*. Stanley that isolated from 5 rope isolates. Three *S.* Derby were from 3 ropes isolates. Three isolates were identified as *S.* Montevideo that isolated from boot covers (3). Two were identified as *S*. Uganda that originated from rope (1) and trim (1). Two *S.* Muenchen that isolated from1 trim isolate and

1 MircoTally isolate. Two *S.* Typhimurium which were from 2 ropes samples. One was identified as *S.*London that isolated from a rope isolate. One *S.* Saintpaul for one rope isolate. Tables 4-4 and 4-5 summarize the distribution of these isolates according to sampling point, treatment, and pig group.

4.4 Discussion

While significant differences were observed during the 6-week (midway) sampling period, the results showed that supplementing market pig finishing diets with BIOPLUS® 2B and Probicon L28 during the finishing period had no effect (P>0.05) on the prevalence of STEC or *E. coli* O26, O121, O45, O103 and O145at loadout when compared with pigs fed a control finishing diet. The *Salmonella* prevalence was too low to detect a treatment effect. Although this study did not demonstrate that the use of Probicon L28 and BIOPLUS® 2B as direct feeding microorganisms in pigs reduced *Salmonella* and STEC in market pigs, there is a large body of research that suggests the use of DFMs pre-harvest can reduce pathogens in animals.

Flach et al. (2022) reported that DFMs in feedlots can reduce the prevalence of foodborne pathogens in feces and peripheral lymph nodes (PLNs) of beef cattle (Flach et al., 2022). Three treatments were used throughout this study, including control, Bovamine, and Probicon L28. Over a five-month period, fecal samples were collected and tested for *E. coli* O157:H7 and *Salmonella,* and results showed a reduction in foodborne pathogens in feces from cattle supplemented with both DFMs, and a significant reduction in the prevalence of *E. coli* O157:H7 and *Salmonella* in feces collected from pens from cattle supplemented with Probicon L28 (Flach et al., 2022*)*.

Lewton et al. (2022) reported on the early health benefits for nursery pigs fed a DFM prepared from multiple strains of *Bacillus subtilis* (DFM). The authors used intestinal mucosa,

plasma immune markers, and intestinal morphology as criteria. Pigs supplemented with the DFM increased the total plasma level of IgA by more than 20% compared with control pigs and increased the villus height of the jejunum (Lewton et al., 2022). These data suggest that nursery pig health can benefit from supplementation with *Bacillus subtilis*.

In another study, pigs treated with BIOPLUS® 2B had lower morbidity and mortality than control pigs throughout the trial period, which was due to a lower incidence of post-weaning diarrhea that was mainly caused by *E. coli* (Alexopoulos et al., 2004). Alexopoulos et al. (2004) also stated that probiotics are not always beneficial to pigs, as they are not a panacea and should be used only after critical thought. Failure in one study doesn't mean probiotics don't work, rather it is important to recognize that experimental designs vary greatly, there are differences in the pathogens and animal species being tested, and variability in immunomodulator system, and farm health can also lead to significant differences in results (Alexopoulos et al., 2004). Future studies with larger sample sizes and higher prevalence of *Salmonella* and STEC may be more effective in determining whether supplementation of BIOPLUS® 2B or Probicon L28 in market pig finishing diets significantly reduces *Salmonella* and STEC in pre-harvest pigs.

In the present study, of the 23 SILN, trim, MicroTally™, and rope samples collected at the abattoir that were positive for STEC, isolates were recovered from 7 samples, and all were characterized as O121 with *stx2e* only (lack *eae*). During an 11-year study, a total of 2,231 *E. coli* isolates from pigs were recovered, 233 of which were *stx2e* producing isolates, and 109 isolates were able to be typed by O-antisera, of which 87 were O139 serogroup. Although attention is often focused on *E. coli* O157, the serogroups that cause edema in post weaning and fattening pigs are often O8, O138, O139, and O147. *Escherichia coli* O157 might be an important serogroup from a public health perspective in humans, but in other animals, other

serogroups may also have significant health implications (Baldo et al., 2020). In another study, from 598 samples collected from 10 pig flows, a total of 178 isolates were recovered and 23 serogroups were identified , among which the three major serogroups were O8, O86 and O121 (Remfry et al., 2021). Of the 178 isolates, 26 isolates carried *stx1a* and 152 isolates carried *stx2e*. Strains isolated with *stx1a,* particularly those associated with *eae*, might cause serious human infections (Remfry et al., 2021). While the STEC subtypes recovered in this study are associated with neonatal diarrhea, post-weaning diarrhea, and edema disease, the authors concluded they were of low public health importance (Remfry et al., 2021).

In the present study, a total of 78 *Salmonella* isolates were recovered from 28 samples. A total of 78 isolates with 13 serotypes were found, *S.* Agona (23), *S.* Senftenberg (12), *S.* Braenderup (9), *S.* monophasic Typhimurium (9), *S.* Worthington (6), *S.* Stanley (5), *S.* Derby (3), *S.* Montevideo (3), *S.* Muenchen (2), *S.* Typhimurium (2), *S.* Uganda (2), *S.* London (1), and *S.* Saintpaul (1). Samples collected at slaughter were associated with more isolates and more serotypes (Table 4-4 and 4-5). There are many serotypes of *Salmonella* that are associated with pigs, but *S*. Choleraesuis and *S*. Typhimurium have been known to cause clinical illness in pigs. In addition, *S*. Agona, *S*. Derby, *S*. Heidelberg and *S*. Infantis serotypes may be associated with mild to moderate diarrhea in pigs (Burrough, 2022). Although *Salmonella* Choleraesuis is the primary cause of most salmonellosis in pigs, infections in other animals and humans are rare (Burrough, 2022), and this serotype was not found in this study.

In 2014, the three most common serotypes of *Salmonella* isolated from pork and pork products in the European Union (EU) were *Salmonella* Typhimurium, *Salmonella* Derby, and *Salmonella* monophasic Typhimurium (Bonardi, 2017). In this study, those three *Salmonella* serotypes were also identified. In 2015, there was an outbreak of *Salmonella* Typhimurium in

North Carolina involving pork barbecues, which infected 280 people and resulted in one death (Clark, 2015). In 2011, outbreaks of *Salmonella* Typhimurium and *Salmonella* Derby that linked to dried pork sausages occurred in several towns in Spain, of which 18% of infected people need medical care (Arnedo-Pena et al., 2016). In 2021, 34 people from 10 states reportedly had infections with the *Salmonella* i4, [5], 12: I:- (Centers for Disease Control and Prevention, 2021). Out of the 27 people interviewed by state and local public health officials, 25 stated that they had consumed or probably consumed Citterio brand Premium Italian-Style Salame Sticks (Centers for Disease Control and Prevention, 2021).

4.5 Conclusion

Supplementation of BIOPLUS® 2B and Probicon L28 in finishing diets of market pigs had no effect on the prevalence of Shiga toxin producing *E. coli* or serogroups O26, O121, O45, O103 and O145 in pig feces, boot covers, ropes, and lymph node samples of commercial pigs at loadout or at the abattoir. *Escherichia coli* O121 with *stx2e* only (lack *eae*) was isolated from SILN samples and is usually associated with edema disease in pigs but has little effect on human health. The prevalence of *Salmonella* throughout the experiment was very low which limited power and the ability to detect a treatment effect, should one exist. Of the *Salmonella* serotypes recovered, *S.* Agona was most frequently isolated, but several serotypes associated with outbreaks in humans were also identified, with the presence of *S.* Typhimurium (including monophasic Typhimurium) perhaps most notable from a public health perspective. Larger sample sizes and/or enrolling animals with a higher prevalence of pathogens, particularly *Salmonella,* would improve power and the ability to determine if BIOPLUS® 2B or Probicon L28 impact pathogen prevalence in market pigs when supplemented into finishing diets.

4.6 Figures and tables

Figure 4-1. Barn Map of Finishing Diets Assigned to Pens of Group 1 Market Pigs (8-10 pigs per pen) During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.

39 BioPlus®2B	22 BioPlus®2B
38 BioPlus®2B	23 BioPlus®2B
37 BioPlus®2B	24 BioPlus®2B
36 Probicon	25 Probicon
35 Probicon	26 Probicon
34 Probicon	27 Probicon
33 Control	28 Control
32 Control	29 Control
31 Control	30 Control

19 Control	2 Control
18 Control	3 Control
17 Control	4 Control
16 BioPlus®2B	5 BioPlus®2B
15 BioPlus®2B	6 BioPlus®2B
14 BioPlus®2B	7 BioPlus®2B
13 Probicon	8 Probicon
12 Probicon	9 Probicon
11 Probicon	10 Probicon

Figure 4-2. Barn Map of Finishing Diets Assigned to Pens of Group 2 Market Pigs (8-10 pigs per pen) During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.

39 Control	22 Control
38 Control	23 Control
37 Control	24 Control
36 Probicon	25 Probicon
35 Probicon	26 Probicon
34 Probicon	27 Probicon
33 BioPlus®2B	28 BioPlus®2B
32 BioPlus®2B	29 BioPlus®2B
31 BioPlus®2B	30 BioPlus®2B

19 BioPlus®2B	2 BioPlus®2B
18 BioPlus®2B	3 BioPlus®2B
17 BioPlus®2B	4 BioPlus®2B
16 Control	5 Control
15 Control	6 Control
14 Control	7 Control
13 Probicon	8 Probicon
12 Probicon	9 Probicon
11 Probicon	10 Probicon

Table 4-1. Treatment Effects in the Analyses of Shiga toxin-producing *Escherichia coli* (STEC), *E. coli* O26 and *E. coli* O121 Prevalence from the Feces of Pigs Fed Control, BIOPLUS® 2B and Probicon Diets for 6 and 13 Weeks During the Finishing Period. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.

Sample Type	Serogroup	Time	Treatment	Pos. Rate (95% Conf. Int.)	Odds Ratio to (P-value)
Pig Feces	STEC	6 wk	Control	22.9% (14.5%,34.1%)	--
			BIOPLUS® 2B	50.0% (37.2%,62.7%)	3.37 (<.001)
			Probicon	36.2% (24.2%,50.2%)	1.91 (0.110)
		13 wk	Control	10.0% (5.3%,18.2%)	--
			BIOPLUS® 2B	5.1% (2.2%,11.2%)	0.48 (0.168)
			Probicon	19.0% (11.1%,30.7%)	2.12 (0.113)
	O26	6 wk	Control	14.3% (8.2%,23.7%)	--
			BIOPLUS® 2B	43.4% (32.0%,55.6%)	4.59 (<.001)
			Probicon	30.1% (20.0%,42.5%)	2.57 (0.025)
		13 wk	Control	3.5% (1.4%,8.9%)	--
			BIOPLUS® 2B	3.3% (1.2%,9.0%)	0.95 (0.939)
			Probicon	8.8% (4.4%,16.9%)	2.65 (0.117)
	O121	6 wk	Control	1.3% (0.3%,5.4%)	--
			BIOPLUS® 2B	9.3% (4.6%,17.8%)	7.96 (0.011)
			Probicon	10.3% (5.2%,19.4%)	8.94 (0.008)
		13 wk	Control	6.2% (2.7%,13.4%)	--
			BIOPLUS® 2B	1.6% (0.4%,6.5%)	0.24 (0.087)
			Probicon	2.9% (1.0%,8.4%)	0.46 (0.253)

Table 4-2.Treatment Effects in the Analyses of STEC Prevalence Status for LN Samples During Harvest Sample Collection. Probicon: A Standard Corn-SBM Finishing Diet with Probicon L28 Supplemented through Water Lines at A Target Concentration of 1.0×10^6 CFU/head/day Using a Water Medicator system. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.

Sample Type	Serogroup	Treatment	Pos. Rate (95% Conf. Int.)	Odds Ratio to (P-value)
LN	STEC	Control	4.5% (2.1%,8.5%)	--
		BIOPLUS® 2B	2.6% (0.8%,5.9%)	0.53 (0.401)
		Probicon	2.2% (0.6%,5.4%)	0.45 (0.289)
	O26	Control	0.0% (0.0%,1.8%)	--
		BIOPLUS® 2B	0.0% (0.0%,1.9%)	NA
		Probicon	0.5% (0.0%,3.0%)	1.02 (0.494)
	O121	Control	3.0% (1.1%,6.5%)	--
		BIOPLUS® 2B	1.0% (0.1%,3.7%)	0.32 (0.276)
		Probicon	0.5% (0.0%,3.0%)	0.17 (0.134)
	O145	Control	1.0% (0.1%,3.6%)	--
		BIOPLUS® 2B	1.0% (0.1%,3.7%)	1.01 (1.000)
		Probicon	1.6% (0.3%,4.7%)	1.60 (0.945)

Table 4-3. STEC Serotypes Isolated from Lymph nodes and Trim from Group 1 and Group 2. BIOPLUS® 2B: A Standard Corn-SBM Finishing Diet Supplemented with BIOPLUS® 2B (5.0×10^8 CFU/pound of Feed; ~3.0×10^9 CFU/head/day). Control: A Standard Corn-SBM Diet.

No. of isolates of STEC O121 with *stx2e* only (lack *eae*) with sample types

	Treatment	Lymph node	Trim
Group 1	Control	39	--
Group 2	Bioplus® 2B	23	5

Table 4-4. *Salmonella* Serotypes Isolated from Pig Feces, Boot Covers, Ropes, Lymph nodes, MicroTally™, and Trim from Group 1. Baseline: Upon Arrival to the Finishing Barn. 6 weeks: Approximately Midway on Trial. 13 weeks: Before Loadout.

		No. of isolates with sample types								
Time	Sample types	*S.* Agona	*S.* Senftenberg	*S.* Braenderup	*S.* monophasic Typhimurium	*S.* Typhimurium	*S.* Uganda	*S.* London	*S.* Montevideo	*S.* Saintpaul
Baseline	Pig feces	3								
	Boot covers	6								
	Ropes									
6 weeks	Pig feces			3						
	Boot covers		3	3						
	Ropes		3							
13 weeks	Pig feces		3							
	Boot covers								3	
	Ropes									
Abattoir	Lymph nodes									
	Microtally	3								
	Trim				3					
	Rope in lairage	2	3		1	2	1	1		1

Table 4-5. *Salmonella* Serotypes Isolated from Pig Feces, Boot Covers, Ropes, Lymph nodes, MicroTally^{TM}, and Trim from Group 2. Baseline: Upon Arrival to the Finishing Barn. 6 weeks: Approximately Midway on Trial. 13 weeks: Before Loadout.

		No. of isolates with sample types							
Time	Sample types	*S.* Agona	*S.* Braenderup	*S.* monophasic Typhimurium	S. Worthington	*S.* Stanley	*S.* Uganda	*S.* Derby	*S.* Muenchen
Baseline	Pig feces								
	Boot covers								
	Ropes								
6 weeks	Pig feces								
	Boot covers				6				
	Ropes								
13 weeks	Pig feces								
	Boot covers								
	Ropes	3				5			
Abattoir	Lymph nodes								
	Microtally	1		5					1
	Trim	2					1		1
	Rope in lairage	3	3					3	

References:

Ainslie-Garcia, M. H., Farzan, A., Newman, J. E., Friendship, R. M., & Lillie, B. N. (2018). *Salmonella* fecal shedding in pigs from birth to market and its association with the presence of *Salmonella* in palatine tonsils and submandibular lymph nodes at slaughter. *Canadian Journal of Veterinary Research*, *82*(4), 249-255.

Alexopoulos, C., Georgoulakis, I. E., Tzivara, A., Kyriakis, C. S., Govaris, A., & Kyriakis, S. C. (2004). Field evaluation of the effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine*, *51*(6), 306-312. https://doi.org/10.1111/j.1439-0442.2004.00637.x

Arnedo-Pena, A., Sabater-Vidal, S., Herrera-León, S., Bellido-Blasco, J. B., Silvestre-Silvestre, E., Meseguer-Ferrer, N., Yague-Muñoz, A., Gil-Fortuño, M., Romeu-García, A., & Moreno-Muñoz, R. (2016). An outbreak of monophasic and biphasic *Salmonella* Typhimurium, and *Salmonella* Derby associated with the consumption of dried pork sausage in Castellon (Spain). *Enfermedades Infecciosas Y Microbiol Clinica*, *34*(9), 544-550. https://doi.org/10.1016/j.eimc.2015.11.016

Ayala, D. I., Cook, P. W., Campos, D. L., Brashears, M. M., den Bakker, H., & Nightingale, K. K. (2017). Draft genome sequence of *Lactobacillus salivarius* L28 isolated from ground beef. *Genome Announcements*, *5*(39). https://doi.org/10.1128/genomeA.00955-17

Baldo, V., Salogni, C., Giovannini, S., D'Incau, M., Boniotti, M. B., Birbes, L., Pitozzi, A., Formenti, N., Grassi, A., Pasquali, P., & Alborali, G. L. (2020). Pathogenicity of Shiga

toxin type 2e *Escherichia coli* in pig colibacillosis [Original Research]. *Frontiers in Veterinary Science*, *7*. https://doi.org/10.3389/fvets.2020.545818

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

Burrough, E. R. (2022). *Intestinal salmonellosis in pigs*. Retrieved June 22, 2023 from https://www.merckvetmanual.com/digestive-system/intestinal-diseases-in-pigs/intestinal-salmonellosis-in-pigs

Centers for Disease Control and Prevention. (2014). *E. coli (Escherichia coli)*. Retrieved May 23, 2023 from https://www.cdc.gov/ecoli/general/index.html

Centers for Disease Control and Prevention. (2021). Salmonella *Outbreak Linked to Salami Sticks*. Retrieved August 3, 2023 from https://www.cdc.gov/salmonella/i45-10-21/details.html

Centers for Disease Control and Prevention. (2022). *Foodborne germs and illnesses*. Retrieved May 27, 2023from https://www.cdc.gov/foodsafety/foodborne-germs.html

Chr Hansen. (2023). *BIOPLUS® 2B*. Retrieved June 5, 2023 from https://www.chr-hansen.com/en/animal-health/cards/product-cards/bioplus-2b

Clark, M. (2015). *Tarheel Q* Salmonella *Outbreak Declared Over With 1 Death, 280 Illnesses*. Food Safety News. Retrieved August 2, 2023 from

https://www.foodsafetynews.com/2015/07/tarheel-q-salmonella-outbreak-over-1-death-280-illnesses/

Colello, R., Cáceres, M. E., Ruiz, M. J., Sanz, M., Etcheverría, A. I., & Padola, N. L. (2016). From farm to table: follow-up of Shiga toxin-producing *Escherichia coli* throughout the pork production chain in Argentina [Original Research]. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00093

Fairbrother, J. M. (2023). *Edema Disease in Pigs*. Retrieved May 27, 2023 from https://www.merckvetmanual.com/generalized-conditions/edema-disease/edema-disease-in-pigs

Flach, M. G., Dogan, O. B., Kreikemeier, W. M., Nightingale, K. K., & Brashears, M. M. (2022). Reduction of pathogens in feces and lymph nodes collected from beef cattle fed *Lactobacillus salivarius* (L28), *Lactobacillus acidophilus* (NP51) and *Propionibacterium freudenreichii* (NP28), commercially available Direct-Fed Microbials. *Foods*, *11*(23), 3834. https://www.mdpi.com/2304-8158/11/23/3834

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/https://doi.org/10.1016/j.resmic.2006.09.009

Kim, S., Frye, J. G., Hu, J., Fedorka-Cray, P. J., Gautom, R., & Boyle, D. S. (2006). Multiplex PCR-Based Method for Identification of Common Clinical Serotypes of *Salmonella*

enterica subsp. *enterica*. *Journal of Clinical Microbiology*, *44*(10), 3608-3615. https://doi.org/doi:10.1128/jcm.00701-06

Larsen, S. (n.d.). *On-farm pathogen control: a priority for today's pig farms*. Retrieved May 27, 2023 from https://www.kemin.com/na/en-us/markets/animal/swine/pathogen-control/resources/on-farm-pathogen-control

Lewton, J. R., Woodward, A. D., Moser, R. L., Thelen, K. M., Moeser, A. J., Trottier, N. L., Tempelman, R. J., & Rozeboom, D. W. (2022). Effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial on immunity markers and intestinal morphology in diets fed to weanling pigs. *Translational Animal Science*, *6*(3), txac083. https://doi.org/10.1093/tas/txac083

Marshall, K. E., MPH1; , Nguyen , T.-A., MPH1; Michael Ablan, MPH1,2; , Nichols, M. C., DVM1; , Robyn, M. P. D., Sundararaman, P. M., Whitlock, L. M., E. Wise, M. P., & A. Jhung, M. M. (2020). *Investigations of possible multistate outbreaks of* Salmonella*, Shiga toxin–producing* Escherichia coli*, and* Listeria monocytogenes *infections — United States, 2016*. Retrieved June 5, 2023 from https://www.cdc.gov/mmwr/volumes/69/ss/ss6906a1.htm

Mesonero-Escuredo, S., Morales, J., Mainar-Jaime, R. C., Díaz, G., Arnal, J. L., Casanovas, C., Barrabés, S., & Segalés, J. (2021). Effect of edema disease vaccination on mortality and growth parameters in nursery pigs in a Shiga toxin2e positive commercial farm. *Vaccines (Basel)*, *9*(6). https://doi.org/10.3390/vaccines9060567

Palkovicsné Pézsa, N., Kovács, D., Rácz, B., & Farkas, O. (2022). Effects of *Bacillus licheniformis* and *Bacillus subtilis* on gut barrier function, proinflammatory response, ROS production and pathogen inhibition properties in IPEC-J2-*Escherichia coli*/*Salmonella* Typhimurium Co-Culture. *Microorganisms*, *10*(5). https://doi.org/10.3390/microorganisms10050936

Pipestone Veterinary Services. (2018). Salmonella *Infection in Pigs*. Retrieved May 23, 2023 from https://pipevet.com/salmonella-in-pigs

Remfry, S. E., Amachawadi, R. G., Shi, X., Bai, J., Tokach, M. D., Dritz, S. S., Goodband, R. D., Derouchey, J. M., Woodworth, J. C., & Nagaraja, T. G. (2021). Shiga toxin–producing *Escherichia coli* in feces of finisher pigs: isolation, identification, and public health implications of major and minor serogroups. *Journal of Food Protection*, *84*(1), 169-180. https://doi.org/https://doi.org/10.4315/JFP-20-329

Romo-Barrera, C. M., Castrillón-Rivera, L. E., Palma-Ramos, A., Castañeda-Sánchez, J. I., & Luna-Herrera, J. (2021). *Bacillus licheniformis* and *Bacillus subtilis*, probiotics that induce the formation of macrophage extracellular traps. *Microorganisms*, *9*(10). https://doi.org/10.3390/microorganisms9102027

Shi, Y., Li, J., Shen, Y., & Sun, Z. (2020). Using probiotics to mute *Salmonella enteric* serovar Typhimurium: an opinion. *Frontiers in Bioengineering and Biotechnology*, *8*, 558. https://doi.org/10.3389/fbioe.2020.00558

Svboda, A. L., & Cutter, C. (2012). STEC: Shiga-toxin producing *Escherichia coli* in pork. https://porkgateway.org/resource/stec-shiga-toxin-producing-escherichia-coli-in-pork/

Chapter 5 - Conclusion

In the first study, a method for BAX® System SalQuant® for quantifying *Salmonella* from pre-harvest and harvest pig samples was developed and validated as a rapid and feasible quantitative method. With the exception of spleen samples, SalQuant® was able to accurately estimate *Salmonella* concentrations within 6 to 10 hours in boot cover, pig feces, and ropes samples. Further studies should examine how to use this method in commercial facilities and establish baselines regarding different levels of *Salmonella* in pork samples (Vargas et al., 2023). Improving the SalQuant® curve for spleens is also a priority.

Results from Flach et al. (2022) showed that concentrations of *E. coli* O157:H7, *Salmonella* and *Clostridium perfringens* were significantly reduced in cow manure collected from cattle fed diets supplemented with Probicon L28, suggesting efficacy as a pre-harvest intervention (Flach et al., 2022). We therefore investigated whether Probicon L28 is an effective pre-harvest intervention for reducing *Salmonella* and Shiga toxin-producing *E. coli* in pigs. In a second experiment, it was demonstrated that supplementation of BIOPLUS® 2B and Probicon L28 in the finishing diet of market pigs had no effect on the prevalence of Shiga toxin producing *E. coli* or serogroups O26, O121, O45, O103, and O145 in pig feces, boot cover, rope, and lymph node samples of commercial pigs (at loadout or at the abattoir). A future study with a larger sample size and a higher prevalence of *E. coli* and *Salmonella* may be more effective in determining whether adding BIOPLUS® 2B or Probicon L28 to the finishing diets of market pigs significantly reduces *E. coli* and *Salmonella*. Research on a large commercial pig farm could be considered as a future experimental direction.

References:

Flach, M. G., Dogan, O. B., Kreikemeier, W. M., Nightingale, K. K., & Brashears, M. M. (2022). Reduction of pathogens in feces and lymph nodes collected from beef cattle fed *Lactobacillus salivarius* (L28), *Lactobacillus acidophilus* (NP51) and *Propionibacterium freudenreichii* (NP28), commercially available Direct-Fed Microbials. *Foods*, *11*(23), 3834. https://www.mdpi.com/2304-8158/11/23/3834

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. https://www.mdpi.com/2304-8158/12/4/822#