Investigation into the effect of in-feed flavophospholipol on Salmonella shedding and antimicrobial resistance in pigs

by

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ABSTRACT

INVESTIGATION INTO THE EFFECT OF IN-FEED FLAVOPHOSPHOLIPOL ON SALMONELLA SHEDDING AND ANTIMICROBIAL RESISTANCE IN PIGS

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The objectives of this study were to investigate the time course of Salmonella shedding and antibody response, as well as determine the impact of in-feed flavophospholipol in reducing Salmonella and antimicrobial resistance in grower-finisher pigs. Over 10 weeks, 45 Salmonella-infected pigs either received feed containing 4 ppm of flavophospholipol or non-medicated diet. Weekly fecal samples, and tissue samples at slaughter were cultured for Salmonella and generic E. coli. A subset of the isolates were tested for antimicrobial susceptibility. In addition, pigs were tested for Salmonella antibodies multiple times. Data were analyzed using a multilevel mixed effects logistic regression model. Flavophospholipol had no impact in reducing Salmonella shedding; however, antimicrobial resistance in E. coli was decreased. Salmonella shedding decreased from 12 to 20 weeks of age ($P<0.05$) during which antibody response increased ($P<0.05$). Eight different Salmonella serotypes were isolated with pigs being reinfected with different serotypes. At slaughter, Salmonella was isolated from some pigs that had not tested positive on weekly fecal checks for $\geq 7$ weeks indicating that the absence of Salmonella in fecal samples may not be indicative of the carcass being Salmonella-free.
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CONTRIBUTIONS

Saranya Nair contributed in the fieldwork, sample collection, and all laboratory works including bacteriology, serogrouping, antimicrobial susceptibility testing, and serogrouping.

Dr. Robert Friendship helped coordinate the fieldwork, interpreted the results, and provided critical feedback on the logistics of the study.

Dr. Vahab Farzan coordinated the laboratory work, helped with data collection and autopsies, assisted with the data analysis and interpreted the results.

Dr. Terri O'Sullivan assisted with the data analysis.

Karen Richardson helped with scheduling vehicles.

Kate Bottoms, Maria Amezcua, Adam Totafurno, Glen Cassar, Bryan Bloomfield, Emily Ardnt, Danielle Hopkins, Heather Reinhardt, Melissa Atkinson assisted with collecting samples and weighing pigs.

Jane Newman helped with data collection and bacterial culturing.

Joyce Rousseau assisted with purchasing laboratory materials and provided technical help in laboratory work when needed.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii
LIST OF TABLES .................................................................................................................. vii
LIST OF FIGURES ............................................................................................................... viii
LIST OF ABBREVIATIONS ................................................................................................. ix

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW ........................................... 1
  1.1 Introduction .................................................................................................................. 1
  1.2 Salmonella .................................................................................................................. 1
    1.2.1 Epidemiology of Human Salmonellosis ............................................................... 2
    1.2.2 Impact of Salmonellosis on Public Health .......................................................... 4
    1.2.3 Salmonella Serotypes Impacting Public Health in Canada ................................ 5
    1.2.4 Salmonella Infection in Swine ........................................................................... 6
    1.2.5 Farm to Fork Transmission of Salmonella in Swine .......................................... 7
    1.2.6 Post-Harvest Control Measures ........................................................................ 9
    1.2.7 Pre-Harvest Control Measures ........................................................................... 10
    1.2.8 Salmonella Serotypes Impacting Swine in Canada ........................................... 14
  1.3 Antimicrobial Resistance ............................................................................................ 16
    1.3.1 Antimicrobial Usage in Canada ........................................................................... 16
    1.3.2 Multi-drug Resistant Salmonella ........................................................................ 17
    1.3.3 Antibiotic Usage on Canadian Swine Farms ....................................................... 18
    1.3.4 Salmonella Typhimurium DT 104 ....................................................................... 19
    1.3.5 Other Resistant Salmonella Serotypes ............................................................... 20
    1.3.6 Prevention and Control Measures for AMR ....................................................... 21
  1.4 Flavophospholipol ....................................................................................................... 22
    1.4.1 Effectiveness on Gram-Positive vs. Gram-Negative Bacteria ............................. 22
    1.4.2 Use of Flavophospholipol ................................................................................... 23
    1.4.3 Impact on Gut Microflora ................................................................................... 23
    1.4.4 Mechanism against Antimicrobial Resistance .................................................. 24
  1.5 Summary ..................................................................................................................... 26
  1.6 References ................................................................................................................... 27
CHAPTER 2: A CLINICAL TRIAL INVESTIGATING THE IMPACT OF IN-FEED FLAVOPHOSPHOLIPOL ON *SALMONELLA* SHELDDING AND ANTIMICROBIAL RESISTANCE IN PIGS

2.1 Abstract ............................................................................................................. 38
2.2 Introduction ........................................................................................................ 38
2.3 Materials and Methods ...................................................................................... 40
2.4 Results ................................................................................................................ 44
2.5 Discussion .......................................................................................................... 46
2.6 References ......................................................................................................... 50

CHAPTER 3: TIME COURSE OF *SALMONELLA* SHELDDING AND ANTIBODY RESPONSE TO *SALMONELLA* IN NATURALLY-INFECTED PIGS THROUGH THE GROWER-FINISHER STAGE UNTIL SLAUGHTER

3.1 Abstract ............................................................................................................. 62
3.2 Introduction ........................................................................................................ 63
3.3 Methods and Material ....................................................................................... 64
3.4 Results ................................................................................................................ 67
3.5 Discussion .......................................................................................................... 70
3.6 References ......................................................................................................... 74

CHAPTER 4: CONCLUSIONS

4.1 Research Summary and Conclusions .................................................................. 81
4.2 References ......................................................................................................... 85

Appendix I ............................................................................................................. 86
Appendix II ........................................................................................................... 94
LIST OF TABLES

Table 2.1: The mean weight (kg) with standard deviation and range for Week 1, Week 4, Week 7 and Week 11 in the flavophospholipol treatment group and the control group ………..52

Table 2.2: The mean average daily gain (kg/day) with standard deviation and range for Week 1 to 4, Week 4 to 7, Week 7 to Week 11 and Week 1 to Week 11 in the flavophospholipol treatment group and the control group ……………………………………………53

Table 2.3: *Salmonella* isolate resistance patterns in treatment (flavophospholipol) and control groups at Week 1 (before treatment), at Week 3 and Week 4 (during treatment) …………………54

Table 2.4: *E. coli* resistance patterns in treatment (flavophospholipol) and control groups at Week 1 (before treatment) and at Week 11 (after treatment) …………………………………………55

Table 2.5: Comparison of resistance and MIC distribution (squashtogram) for *Salmonella* isolates recovered from flavophospholipol treated (Tx; n=30) (n=isolates) and control (C;n=30) pigs at Week 1 and Week 3…………………………………………………………………………56

Table 2.6: Comparison of resistance and MIC distribution (squashtogram) for *Salmonella* isolates recovered from flavophospholipol treated (Tx; n=10) and control (C;n=14) pigs at Week 1 and Week 4……………………………………………………………………………………57

Table 2.7: Comparison of resistance and MIC distribution (squashtogram) for *E. coli* isolates recovered from flavophospholipol treated (Tx; n=36) and control (C; n=30) pigs at Week 1 and Week 11……………………………………………………………………………………58
LIST OF FIGURES

Figure 2.1: *Salmonella* shedding in pigs in the treatment (flavophospholipol) and control group from Week 1 to Week 11………………………………………………………………………………59

Figure 2.2: *Salmonella* status in pens in the treatment (flavophospholipol) and control group from Week 1 to Week 11………………………………………………………………………60

Figure 2.3: Survival probability (time to event: when pigs stop shedding *Salmonella*) of the treatment (flavophospholipol) group versus the control group from Week 1 to Week 11……61

Figure 3.1: This chart illustrates the pig’s *Salmonella* status (determined by *Salmonella* culturing) from the beginning of the trial at Week 1 (W1) to Week 11 (W11) and at slaughter (SH) based on how many times the pig was positive (counts) by trend of *Salmonella* shedding grouped together………………………………………………………………………………77

Figure 3.2: The prevalence of *Salmonella* shedding from Week 1 (W1) to Week 11 (W11) versus the mean level of serum adjusted optical density (OD) obtained by ELISA in pigs at Week 1, 4, 7 and 11 (adjusted optical density represents the amount of antibody against *Salmonella* in serum samples)………………………………………………………………………………78

Figure 3.3: Kaplan-Meier Survival Curve illustrates the survival probability of *Salmonella* shedding over 10 weeks with the time to event of when pigs stop shedding *Salmonella* (last positive culture)………………………………………………………………………………79

Figure 3.4: Predictive probability of *Salmonella* shedding in pigs constructed against serum adjusted optical density (OD) obtained by ELISA (adjusted OD represents the amount of antibody against *Salmonella* in the serum samples)……………………………………………………80
LIST OF ABBREVIATIONS

ADG – average daily gain
AMP – ampicillin
AUG – amoxicillin/clavulanic acid
AXO – ceftriaxone
CHL – chloramphenicol
CIP – ciprofloxacin
FIS – sulfisoxazole
FOX – cefoxitin
GEN – gentamicin
NAL – nalidixic acid
OD – optical density
STR – streptomycin
SXT – trimethoprim/sulphamethoxazole
TET – tetracycline
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

*Salmonella*, a genus of bacteria that can potentially cause enteric illness, is a public health and food safety concern. In Canada salmonellosis is estimated to affect approximately 88,000 people, annually (Thomas et al., 2013). Furthermore, the emergence of multi-antimicrobial resistant *S. Typhimurium*, the most common strain of the bacteria found on Ontario swine farms over the past two decades, is alarming. Monitoring and finding better on-farm control for *Salmonella* is important in order to reduce the risk of *Salmonella* spp. moving from farm to retail pork via contamination at slaughter. Research suggests flavophospholipol, an antibiotic with nontransferable resistance, can reduce *Salmonella* shedding, possibly by altering the gut microflora in favour of beneficial bacteria inhibiting the colonization of *Salmonella* (Bolder et al., 1999; Butaye et al., 2003; Dealy and Moeller, 1976; Dorr and Gebreyes, 2009; Van den Bogaard et al., 2002) and can improve growth performance and feed efficiency (Butaye et al., 2003; Pfaller, 2006). The “plasmid-curing” properties (Van den Bogaard et al., 2002) of flavophospholipol may also have the ability to reduce antimicrobial resistance in *Salmonella* (Dealy and Moeller, 1976; Pfaller, 2006; Van den Bogaard et al., 2002). Monitoring the trends of *Salmonella* and investigating the influence of flavophospholipol on *Salmonella* in swine is beneficial for disease control and prevention.

1.2 *Salmonella*

*Salmonella* spp. belong to the *Enterobacteriaceae* family (Kauffmann, 1961). It is a Gram-negative facultative anaerobic bacterium distinguished by O, H and Vi antigens (Giannella,
1996; Gray et al, 1996; Grimont and Weill, 2007). The pathogen colonizes in the gastrointestinal tract resulting typically in self-limiting gastroenteritis and seldom enteric fevers or bacteremia (Giannella, 1996). Having diverged over 100 million years ago from *Escherichia coli* (Ochman and Wilson, 1987), *Salmonella* has developed approximately 2,600 identified serotypes based on the Kauffmann-White Scheme (Grimont and Weill, 2007; Kauffmann, 1961).

Salmonellosis can be divided into typhoidal, restricted to humans, and non-typhoidal, affecting both humans and animals (Langridge et al, 2012). Over the last decade, there has been a drastic decline in typhoidal salmonellosis due to better living conditions, sanitation and health care (Rabsch et al., 2013), however typhoidal salmonellosis continues to be very common in developing nations (Chimalizeni et al, 2010). Non-typhoidal salmonellosis with approximately 1.3 billion cases annually (Chimalizeni et al., 2010; O’Ryan, Prado, and Pickering, 2005), is highly prevalent in industrialized countries around the world and is one of the leading enteric illnesses in Canada (Rabsch et al., 2013). There are over 2,000 serotypes of non-typhoidal *Salmonella* (Public Health Agency of Canada, 2015) including various multi-drug resistant serotypes such as *S. Typhimurium* DT 104 (hereafter referred to as DT 104).

### 1.2.1 Epidemiology of Human Salmonellosis

*Salmonella* spp. can spread through fecal-oral transmission between hosts (Stevens and Gray, 2013). Transmission can occur through various methods: consumption of contaminated food and water, unsanitary handling of food, spread of the pathogen by infected animals, through animal feed, unclean surfaces, by fomites etc (Bäumler et al, 2000; Giannella, 1996; Rabsch et al., 2013). In infected individuals, the colonization of *Salmonella* in the gastrointestinal tract can
result in symptoms such as diarrhea, fever, abdominal cramps, nausea and vomiting (Giannella, 1996). Symptoms may last anywhere from 2 to 7 days within 6 to 48 hours after infection and the individual may remain infectious for days up to weeks post-infection (Giannella, 1996). *Salmonella* carriers, infected individuals who do not show clinical symptoms (asymptomatic), are also capable of transmitting the disease to others (Giannella, 1996; Rabsch et al., 2013). The carrier state, common in animals, causes them to shed *Salmonella* persistently or intermittently without showing signs of the disease (Bronze and Greenfield, 2005; Verbrugghe et al., 2011).

The majority of individuals do not require medication in order to alleviate gastroenteritis caused by the pathogen as it is self-limiting and thus often under-reported (Public Health Agency of Canada, 2015). More critical patients based on clinical symptoms, whether they have bacteremia or enteric fever, may require antibiotics (Giannella, 1996). Of the pathogens estimated to cause the greatest number of hospitalizations in Canada, nontyphoidal *Salmonella* places second after norovirus with an estimated 925 hospitalizations and 17 deaths annually (Thomas et al., 2015). Often very young children (under 6 months of age), individuals with compromised immune systems and the elderly (over 65 years of age) are most likely to have complications and become hospitalized from contracting *Salmonella* (Public Health Agency of Canada, 2015). Studies have also found that hospitalization and mortality are greater in individuals who contract multi-drug resistant DT 104 (Martin et al., 2004; Travers and Barza, 2002).
1.2.2 Impact of Salmonellosis on Public Health

There is an estimated 4 million cases of domestically acquired foodborne illness in Canada every year of which non-typhoidal salmonellosis is one of the four leading diseases that are collectively responsible for 90% of enteric illnesses annually (Thomas et al., 2013). Other leading pathogens include norovirus, *Clostridium perfringens* and *Campylobacter* spp. (Thomas et al., 2013). Non-typhoidal *Salmonella* accounts for estimated 87,510 cases annually, ranking as the 4th leading enteric pathogen in Canada (Thomas et al., 2013). The United States also presented with the same four leading pathogens responsible for 90% of enteric illnesses (Scallan et al., 2011). These Canada and US estimations were consistent with those reported in other countries; non-typhoidal *Salmonella* was a leading foodborne pathogen in United States, Australia, New Zealand, France, UK and Greece (Adak et al., 2002; Cressey and Lake, 2011; Gkogka et al., 2011; Hall et al., 2005; Thomas et al., 2013; Vaillant et al., 2005).

Non-typhoidal *Salmonella* is transmitted to humans in various ways but significantly due to foodborne transmission. According to the Foodborne Illness in Toronto report by Toronto Public Health, the average proportion of foodborne transmission from 1998 to 2007 of *Salmonella* was 89% (Toronto Public Health, 2009). Salmonellosis outbreaks associated to food products occur frequently through the year. Foodborne transmission of *Salmonella* is particularly high in meats, poultry, dairy products and egg products. Between 1998 and 2007, salmonellosis was also found to contribute to 554 reported cases annually making it the 2nd most sporadic foodborne pathogen in Toronto (Toronto Public Health, 2009). In Ontario, 23% of enteric illnesses from 1997 to 2001 have been attributed to *Salmonella* spp. (Lee and Middleton, 2003; Toronto Public Health, 2009). The Laboratory Surveillance Data for Enteric Pathogens in
Canada 2006 Summary published by Public Health Agency of Canada revealed that Ontario had the largest number of reported salmonellosis cases (n=2697) in Canada while Quebec (n=1078) and British Columbia (n=735), respectively followed with less than half the number of cases compared to Ontario (Public Health Agency of Canada, 2006). However, the number of cases is likely much higher as salmonellosis is believed to be under-reported.

1.2.3 Salmonella Serotypes Impacting Public Health in Canada

There are over 2,600 serotypes of Salmonella enterica, identified based on the combination of O and H antigens (Grimont and Weill, 2007; Kauffmann, 1961). The O antigen is distinguished by its chemical makeup and is found on the bacterial surface covering (outermost portion of the lipopolysaccharide) while the H antigen is the protein content found in the flagella (Giannella, 1996; Grimont and Weill, 2007; Wain and Olsen, 2013). Differences in the combination of O and H antigens can make certain serotypes more potent leading to severe symptoms while making other serotypes responsible for milder symptoms (Rycroft, 2000). A prime example of this would be the increased virulence of S. Typhimurium serotype, containing the O4 and O5 antigens, compared to S. Typhimurium var. Copenhagen serotype, which only contains the O4 antigen (Rycroft, 2000).

The non-typhoidal Salmonella serotypes having the greatest impact on Canadian public health and contributing to more than 50% of human salmonellosis in 2006 were S. Enteritidis (23%), S. Typhimurium (17%) and S. Heidelberg (12%) (Public Health Agency of Canada, 2006). In 2013, these serotypes remained the top three in all of Canada with an increase in S. Enteritidis (24%) and S. Heidelberg (13%) and a decrease in S. Typhimurium (12%) (Public
Health Agency of Canada, 2013a, 2013b). The most common S. Enteritidis phage types were DT 8 (38%), DT 13a (15%) and DT 13 (9%) while the most common ones recovered from S. Heidelberg were DT 19 (44%), DT 29 (26%) and DT 26 (4%) (Public Health Agency of Canada, 2013a). The most prevalent S. Typhimurium phage types in Canada in 2013 were DT 10 (12%), DT 104 (12%) and DT 108 (10%) (Public Health Agency of Canada, 2013a).

1.2.4 Salmonella Infection in Swine

Swine are considered important reservoirs of non-typhoidal Salmonella (Gray and Fedorka-Cray, 1996; Letellier et al., 2000; Stevens and Gray, 2013). Salmonellosis manifests by colonization in the gut of swine resulting in persistent or intermittent shedding of Salmonella in feces (Stevens and Gray, 2013). Occasionally, colonization of Salmonella is seen in the lymph nodes and other tissues (Groves et al., 1970; Scherer et al., 2008; Stevens and Gray, 2013). The clinical signs of salmonellosis in pigs, depending on the serotype, include watery, yellow diarrhea, lack of appetite, cough, dehydration, fever and fatigue (Gray and Fedorka-Cray, 1996; Scherer et al., 2008; Stevens and Gray, 2013). S. Choleraesuis and S. Typhimurium are the most closely associated with clinical disease, in particular with septicemia and enterocolitis, respectively (Gray and Fedorka-Cray, 1996; Stevens and Gray, 2013).

Salmonellosis impacts swine differently based on age, seasonality and serotype. Studies have found that suckling pigs are less likely predisposed to Salmonella infections due to lactogenic immunity provided to them from their dam (Stevens and Gray, 2013). However, once the pigs are weaned this immunity is absent. Therefore, Salmonella prevalence greatly increases during the nursery stage and may remain high through the early grower stage but generally is
barely detected before slaughter (Kranker et al., 2003). In addition to the disappearance of lactogenic immunity, this increase in salmonellosis prevalence in the nursery is perhaps a result of intensive confinement and modern husbandry practices (Andres and Davies, 2015; Kranker et al., 2003). The integration of various litters in the nursery, change in feed and the lack of antibodies piglets would have received from the sow’s milk can lead to an increased exposure to the pathogen at a time of low piglet immunity (Kranker et al., 2003). Mortality tends to be higher in younger pigs than older pigs, however morbidity remains constant between pigs of all ages (Stevens and Gray, 2013).

The trend of *Salmonella* infection amongst swine also depends on the *Salmonella* serotype. *Salmonella Typhimurium* predominantly infects pigs from 6 to 12 weeks of age (Stevens and Gray, 2013). When age-matched weaned piglets were inoculated with oral dosing (10^8 CFU) of *S. Typhimurium* and *S. Choleraesuis*, piglets that were infected with *S. Typhimurium* showed rapid clinical signs of fever, diarrhea and vomiting; however, clinical signs reduced within 2-3 days (Watson et al., 2000). Meanwhile, piglets that were infected with *S. Choleraesuis* had prolonged clinical signs with increased temperatures, intermittent diarrhea and fatigue (Stevens and Gray, 2013; Watson et al., 2000). The trend of *Salmonella* infection can also be impacted by seasonality; *Salmonella* seroprevalence was increased when sampling during summer as opposed to winter (Hautekiet et., 2008).

1.2.5 Farm to Fork Transmission of *Salmonella* in Swine

A multi-drug resistant *Salmonella* outbreak not only impacts productivity and causes economic losses for swine producers but also can pose a risk to public health (Farzan and
Friendship, 2010). *Salmonella* is commonly recovered from swine farms; Farzan et al. (2008a) reported *Salmonella* from 46% of Ontario swine farms (37 of 80 farms). Furthermore, *Salmonella* was recovered from 18% of 4441 samples collected from grower-finisher pigs in Canada between 2006 to 2013 (Public Health Agency of Canada, 2015b). The increased prevalence of *Salmonella* in swine is becoming concerning to food safety as it can be passed through the food chain to humans given the pork is not cooked thoroughly or due to cross contamination of crops, produce and groundwater with swine manure. Although the proportion of human salmonellosis attributable to pork consumption is unknown in Canada, it is approximately 6-9% and 1.2% in the US (Frenzen et al., 1999) and Denmark (Alban et al., 2012).

*Salmonella* infections and transmission can occur at various stages through pork production: at primary production sites, in the environment through ground water, improper manure management, through cross-contamination at slaughterhouses and during pork processing (De Ridder et al., 2013). Research has revealed there is a greater prevalence of *Salmonella* in slaughterhouses in comparison to pig farms (Hurd et al, 2004). Often, the pigs are *Salmonella* asymptomatic/carriers and shed *Salmonella* in high stress environments for example at weaning, or when shipping to slaughterhouses. This can potentially harm the Canadian pork industry and exporting to foreign markets due to the high risk of transmission of *Salmonella* from the food production chain to humans. Prevention of farm to fork transmission of *Salmonella* needs to start at the farm level in order to reduce contamination during slaughter.
1.2.6 Post-Harvest Control Measures

During transport to slaughterhouses and waiting in holding areas prior to slaughter, the increase in stress and spike in cortisol induces heightened colonization and shedding of *Salmonella* in pigs (Verbrugghe et al., 2011). A great deal of cross-contamination, amongst *Salmonella* negative and asymptomatic carriers, also occurs during transport (Berends et al., 1996) and in the holding area (Morgan et., 1987). Research has revealed that there is a greater degree of *Salmonella* shedding and infection when pigs are transported to a slaughterhouse less than 1 km away (Barcelo and Marco, 1998). Whereas, if the slaughterhouse is more than 5 km away there is less risk of infection or shedding (Barcelo and Marco, 1998). In large-scale slaughterhouses, pigs from various farms are mixed together in a holding area. This results in an increase in stress caused by the new environment, the long waiting period, fighting amongst pigs, and the lack of food and water. Studies have also revealed that overnight lairage helps to reduce the colonization of *Salmonella* prior to slaughter (Davies et al., 1999; Warriss et al., 1998).

During slaughter various control measures are used to prevent contamination, like scalding and singeing (Gill and Bryant, 1993). Any surviving *Salmonella* on the pigs after the singeing process can contaminate the equipment (Gill and Bryant, 1993). However, the majority of the contamination occurs when digestive tracts of infected pigs are exposed to the carcass itself or others or digestive tract content contaminates the environment (Belœil et al., 2004). Control measures used to reduce the contamination of *Salmonella* in slaughterhouses include: open flame, boiling water, cleaning the equipment between each carcass, and disinfection of boots and overalls before entering and exiting the slaughter room. Other methods of decontamination of carcass include washing with lactic acid (van Netten et al., 1995), water
(Larsen et al., 2003) and treatment with chlorine (Fabrizio and Cutter, 2004). Although various control measures for preventing *Salmonella* transmission at slaughterhouses are in place, in order to prevent farm to fork transmission, better farm practices to control *Salmonella* are required.

1.2.7 Pre-Harvest Control Measures

Various on-farm interventions exist to help control and prevent *Salmonella* prevalence: biosecurity, feed/water acidification, feed management, antimicrobials, manipulation of gut flora, vaccinations as well as combining more than one of these interventions (Friendship et al., 2009; Friendship - Unpublished Summary Card). These on-farm interventions need to be tailored to the needs of a farm; there is no universal protocol that can be applied to all farms (Andres and Davies, 2015). Each farm and situation is different. Thus, on-farm control measures for *Salmonella* begin with assessing the risk factors and developing an appropriate protocol (Andres and Davies, 2015).

Biosecurity is an emphasized way in reducing the spread of *Salmonella* on farms, however it has not been an adequate means for controlling and preventing *Salmonella*. Biosecurity is the idea of implementing hygiene and sanitation to meet the needs of a particular farm in order to prevent and control the spread of pathogens and improve productivity (Andres and Davies, 2015). The farm type and location, husbandry practices, genetics of pigs, stocking density, size of the farm and the number of neighbouring farms vary from farm to farm (Andres and Davies, 2015; Stevens and Gray, 2013). Therefore, biosecurity is an ongoing process that needs to be tailored to the particular needs of the farm (Andres and Davies, 2015). This entails,
but is not limited to, pest control, limited entry of visitors or controlled entry such as a Danish entry system, prevention of the spread of pathogens from trucks, disinfection of fomites, cleaning and disinfection of barns, feed management, isolation/quarantine areas for new incoming or sick pigs, avoiding high stocking density and depopulation (Stevens and Gray, 2013). Some specific biosecurity measures that have been found helpful include using a hygienic-lock facility, boot baths, all-in all-out (AIAO) housing systems, and disinfection (Hautekiet et al., 2008). Increasing the number of sows, decreasing the floor space for pigs (Hautekiet et al., 2008), not cleaning the pit under slatted floors in farrowing rooms and less than once a day removal of sow dung during lactation period (Belœil et al., 2004) are risk factors associated with increased prevalence and transmission of *Salmonella*.

Various studies have shown that disinfection and sanitation help to keep pigs *Salmonella*-free (Andres and Davies, 2015; Fablet et al., 2005; Friendship et al., 2009; Hautekiet et al., 2008; Stevens and Gray, 2013; Van der Wolf et al., 2001). Batch farrowing and all-in/all-out (AIAO) housing systems, coupled with effective cleaning and disinfection, have been effective in controlling *Salmonella* infection in pigs and cross-contamination in the environment (Andres and Davies, 2015; Hautekiet et al., 2008; Stevens and Gray, 2013). Although various studies have found using an AIAO system along with disinfection as an effective method (Andres and Davies, 2015; Belœil et al., 2004; Fablet et al., 2005; Gotter et al., 2012; Hautekiet et al., 2008; Lo Fo Wong et al., 2004; Van der Wolf et al., 2001), other studies have found no difference between AIAO and continuous flow (Davies et al., 1997; Proescholdt et al., 1999; Rajić et al., 2007; Stege et al., 2001).
In conjunction with biosecurity, it is also useful to monitor the *Salmonella* status of the swine herd and seek to prevent the introduction of serotypes with increased virulence like *S. Typhimurium* (Andres and Davies, 2015). Research has revealed that even after repeated depopulations, disinfections and repopulations, multi-drug resistant *S. Typhimurium* can remain in the environment for many years (Davies et al., 2003). Scandinavian countries, like Denmark have attempted to eradicate the DT 104 strain by depopulation of affected swine herds however were unsuccessful, possibly due to the strain being sub-clinically present in a large proportion of the animals (Alban et al., 2002; Anon, 2000). Eradication of *Salmonella* is unlikely because of the persistence of the pathogen in the environment and also because of the high likelihood of the pathogen being reintroduced (Friendship et al., 2009).

Although biosecurity is capable of reducing *Salmonella* prevalence in pigs and the environment, it cannot sustain a *Salmonella*-free environment alone (Mannion et al., 2007). When paired with other treatments/interventions suitable for the farm, better results are achieved. Different antibiotic treatments have been used to treat *Salmonella* infection, however they have not been successful in eliminating the infection but merely reducing the shedding of *Salmonella* (Stevens and Gray, 2013). A review by Friendship et al. (2009) revealed studies on antimicrobials conducted in the 1970s had positive results, however all the recent studies have shown inconsistent results. Also, the use of antimicrobials for treatment can be detrimental with the increasing antimicrobial resistance amongst *S. Typhimurium* (Friendship et al., 2009).

In terms of vaccinations, live attenuated, orally administered *Salmonella* vaccines have been the most effective against *Salmonella* infection; inactivated vaccines, although readily available in many countries, have not been as effective (Boyen et al., 2008; Stevens and Gray,
A clinical trial comparing three groups that received either S. Typhimurium vaccine, S. Cholerasuis vaccine and or a placebo (control) revealed that the vaccinated groups had decreased *Salmonella* shedding twice as much as the control group over the entire production phase (Farzan and Friendship, 2010). Various other studies have also reported decreased clinical signs with vaccination (De Ridder et al., 2013a; De Ridder et al., 2013b; Springer et al., 2001).

Research has found feed management an effective way of controlling *Salmonella* (Andres and Davies, 2015). In particular, using wet/liquid (Belœil et al., 2004; Farzan et al., 2006; Van der Wolf et al., 2001) and coarse feed (Friendship et al., 2009) are beneficial in reducing shedding. A study conducted in Ontario found coupling liquid feeding with AIAO housing was successful in lowering *Salmonella* shedding (Farzan et al., 2006). However, there are concerns regarding how this would impact productivity because finely ground feed is associated with improved feed efficiency (Andres and Davies, 2015). Acidification of water/feed with organic acids to reduce the pH in intestines and the prevalence of *Salmonella* in the water and feed is also capable of aiding in reducing *Salmonella* (Andres and Davies, 2015). However, results are not consistent on the effectiveness of this method (Andres and Davies, 2015; Friendship et al., 2009).

Various other methods, like the use of electrolytes to prevent dehydration, have been used to alleviate the clinical signs and help in recovery (Stevens and Gray, 2013). The use of sodium chlorate has also been reported to be beneficial in reducing *Salmonella*, but again results are inconsistent (Friendship et al., 2009). The use of probiotics to manipulate the gut microflora to help reduce *Salmonella* has produced inconsistent results (Friendship et al., 2009). The use of prebiotics, essential oils, spray dried porcine plasma, bacteriophages and egg-yolk
immunoglobulin are all methods yet to be convincingly proven in clinical trials (Friendship et al., 2009).

1.2.8 *Salmonella* Serotypes Impacting Swine in Canada

The most prevalent serotype of *Salmonella* found on Canadian swine farms is *S. Typhimurium* (Public Health Agency of Canada, 2006); this is evident when analyzing the frequency of *Salmonella* serotypes over the past two decades. The most common *Salmonella* serotype in swine in Ontario based on isolate submissions to Animal Health Laboratory from 1991 to 2001 was *S. Typhimurium* followed by *S. Brandenburg*, *S. Derby* and *S. Heidelberg* (Zhang et al., 2005). Meanwhile, the overall frequency of *Salmonella* serotypes from the Laboratory for Foodborne Zoonoses also in Ontario, analyzed from 1992 to 2001, found *S. Typhimurium*, followed by *S. Infantis* and *S. Agona* as the most common serotypes (Zhang et al., 2005). Furthermore, between 2002 and 2006 there was an increase in the prevalence of *S. Typhimurium* amongst swine from 20% in 2002 to 49% in 2006 (Public Health Agency of Canada, 2006).

A study conducted in 2004 on 80 grower-finisher pig farms in Ontario found the most common serotypes to be *S. Typhimurium* var Copenhagen, *S. Infantis*, *S. Typhimurium*, *S. Derby*, *S. Agona*, *S. Havana*, and *S. enterica* subsp. I:Rough-O (Farzan et al., 2008a). The most prevalent phage types recovered were DT 104, DT 104a and DT 104b (Farzan et al., 2008a).

Another study conducted on 113 Ontario swine farms, visited 5 times from 2001 to 2006, found *S. Typhimurium* var. Copenhagen (39%) as the most common serotype followed by *S. Typhimurium* (11%), *S. Derby* (10%), *S. Infantis* (7%) and *S. Brandenburg* (5%) (Farzan et al.,
The most common phage types were DT 104, followed by DT 104a and DT 104b (Farzan et al., 2008b). Based on these various studies, S. Typhimurium var Copenhagen and S. Typhimurium are the most prevalent serotypes amongst swine in Ontario, while S. Typhimurium is also one of the top three most common strains amongst humans. Research also shows S. Typhimurium is the most prevalent strain in Quebec as well (Letellier et al., 2000; Letellier et al., 1998).

Meanwhile, research in Alberta and Saskatchewan found S. Derby as the most prevalent serotype followed by S. Typhimurium var Copenhagen. In a study conducted on 10 farrow-to-finish farms in Saskatchewan and Alberta, the most common serotypes were S. Derby (28.5%), S. Typhimurium, var Copenhagen (19.1%), S. Putten (11.8%), S. Infantis (6.8%) and S. Mbandaka (6.1%) (Wilkins et al., 2010). In another study conducted in Alberta on 90 finishing swine farms over a 5-month period found the most common serotypes to be S. Derby (22%), S. Typhimurium var. Copenhagen (18.3%), S. Infantis (14.6%), S. California (7.5%), S. Typhimurium (5.9%), S. Enteritidis (16%), S. London (4.7%), S. Agona (4.7%), S. Mbandaka (3.4%) and others (14.9%) (Rajić et al., 2005).

The prevalence of Salmonella on swine farms is detrimental to public health and food safety. Furthermore, the emergence of the resistant strain S. Typhimurium can promote antimicrobial resistance when transmitted to humans (Alban et al., 2002). Salmonella Typhimurium DT 104 is also associated with increased hospitalization, mortality and economic impact (Farzan, et al., 2008c; Martin et al., 2004; Travers and Barza, 2002). In order to prevent the transmission of Salmonella from swine to humans, better on-farm interventions are needed to minimize the prevalence of Salmonella at slaughter.
1.3 Antimicrobial Resistance

The invention of antibiotic/antimicrobial agents, substances with the ability of destroying or inhibiting microbes (Public Health Agency of Canada, 2015), has been a cornerstone in human and animal medicine in helping keep diseases at bay and patients healthy (Wassenaar, 2005). However microbes respond to antimicrobials by changing their biological makeup to develop resistance. Over time these resistant microbes multiple in the environment leading to new strains that are partially/completely resistant to antimicrobial treatment. Furthermore, many pathogens have formed resistance against more than one group of antimicrobials. Infections that were once treatable with commonly used antibiotics now require more alternative antimicrobials due to the emergence of resistance (Public Health Agency of Canada, 2015).

1.3.1 Antimicrobial Usage in Canada

Antimicrobial resistance is often found where the antimicrobial use is the highest, for example in medical and veterinary practices (Public Health Agency of Canada, 2015). Usage of medically-important antibiotics in Canada in 2013 was approximately 1.4 million kg of which 78% was used in animal production, 21% for human medicine, 1% for companion animals and less than 1% for crops (Public Health Agency of Canada, 2015). When accounting for the population and weight, there was 1.4 times more antimicrobial usage in animal production in comparison to human medicine in 2013 (Public Health Agency of Canada, 2015). Also in 2013, Canada ranked 21 out of 27 countries in the European Surveillance of Veterinary Antimicrobial Consumption report of outpatient antimicrobial use, which is 42 times more than Norway with the least antimicrobial usage (Public Health Agency of Canada, 2015).
Antimicrobial agents are being used in animal production to help prevent diseases, to treat diseases and to improve feed efficiency and growth promotion (Public Health Agency of Canada, 2015). The use of antibiotics as growth promoters is banned in Europe and in legislation is underway to do the same thing in North America. The overall use of antibiotics in animals has decreased in the past decade (Public Health Agency of Canada, 2015). The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) is responsible for monitoring the antimicrobial usage and resistance in humans, animals and retail food for Salmonella and other resistant pathogens and has shown very little change in the antimicrobial resistance pattern of commensal E. coli isolated from swine over the past decade (Public Health Agency of Canada, 2015).

1.3.2 Multi-drug Resistant Salmonella

Salmonella has developed antimicrobial resistance over time (Glynn et al., 1998). There are approximately 100,000 drug resistant non-typhoidal Salmonella infections annually in the United States out of the total 1.2 million Salmonella infections (Centers for Disease Control and Prevention, 2013). The CDC currently places drug resistant non-typhoidal Salmonella under hazard level serious, being a significant antibiotic-resistant threat to public health if not under surveillance in the United States (Centers for Disease Control and Prevention, 2013). A large number of these infections are due to foodborne transmission. Multi-drug resistant Salmonella is prevalent amongst swine. The transmission of resistance from swine to humans, through consumption of contaminated meat, ground water, produce or crops, is an important public health concern. With the increase in antimicrobial resistance to conventional antibiotics (ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole), extended-spectrum
cephalosporin and fluoroquinolones are now being used to treat salmonellosis in humans. Although the conventional medical approach to salmonellosis is to not use antibiotics (Boyen et al., 2008; Chiu et al., 2004; Poppe et al., 1998), resistance is also building against these antimicrobial agents (Chiu et al., 2004; Chiu et al., 2002).

### 1.3.3 Antibiotic Usage on Canadian Swine Farms

Antimicrobial agents are administrated, typically in-feed or through water, to a group of pigs rather than to an individual pig due to the large herd sizes (Friendship, 2000), while injectable antibiotics are usually given to individual sick pigs (Rajić et al., 2006). Antibiotic usage on swine farms is to help prevent disease, to treat disease or to improve growth performance. The Canadian Antimicrobial Resistance Surveillance System 2015 report revealed of the participating grower-finisher pig farms 11% were using antibiotics for disease treatment, 47% for prevention and 36% for increased production. In terms of disease prevention and treatment, it was primarily for enteric (lincomycin and tylosin) and respiratory (chlortetracycline) diseases (Public Health Agency of Canada, 2015). Lincomycin, tylosin, chlortetracycline along with ionophores were antibiotics also being used for growth production (Public Health Agency of Canada, 2015). Furthermore, research by Rajić et al. (2006) analyzed the use and administration of antibiotics on 90 swine farms in Alberta and found chlortetracycline/sulfamethazine/penicillin combination and tylosin most commonly used in-feed for nursery and grower-finisher pigs, respectively. Penicillin was also a common antibiotic used in-water and as an injectable on swine farms (Rajić et al., 2006). In addition, carbadox, apramycin, ceftiofur, neomycin, spectinomycin and trimethoprim-sulfamethoxazole may be used for treatment of clinical *S. Cholerasuis* infection (Friendship, 2000; Winkleman, 1997). Other
commonly used antibiotics on swine farms at the time of these studies included: dimetridazole, lincomycin/spectinomycin, oxytetracycline, tilmicosin, tiamulin, virginiamycin, neomycin/tetracycline and penicillin/streptomycin (Rajić et al., 2006). Dimetridazole and carbadox are no longer used in Canada for livestock, and sulfa drugs are rarely used because of concerns of accidental residues.

1.3.4 *Salmonella Typhimurium DT 104*

Of the *Salmonella* serotypes, the emergence of virulent multi-drug resistant DT 104, commonly found on Ontario swine farms and at slaughter (Farzan et al., 2008a; Farzan et al., 2008c), has been heavily reported in Europe, United States and Canada (Larkin et al., 2006; Poppe et al., 1998; Sandvang et al., 1998; Weese et al., 2001). DT 104, first isolated in the 1980s, is a sub lineage of serotype *S. Typhimurium* (Threlfall et al., 1994). DT 104 was initially found to have penta-resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su) and tetracycline (T) (R-type ACSSuT) (Farzan et al., 2008c; Gebreyes et al., 2004; Glynn et al., 1998; Larkin et al., 2006; Poppe et al., 1998; Weese et al., 2001). However, in a recent study by Farzan et al (2008c) analyzing the resistance patterns of *S. Typhimurium* recovered from Ontario swine farms revealed resistance to various other antibiotics: sulfonamides (Su; 100%), ampicillin (A; 99%), streptomycin (S; 99%), spectinomycin (Sp; 97%), chloramphenicol (C; 96%), tetracycline (T; 93%), florfenicol (F; 93%), neomycin (N; 39%), kanamycin (K; 38%), nitrofurantoin (Ni; 6%) and gentamicin (G; 4%). Resistance pattern “ACSpSSuT” was the most common amongst isolates (88%), while “ACFSpSSuT” and “ACFKNSpSSuT” represented 56% and 26% of isolates respectively (Farzan et al., 2008).
The pathogen developing resistance is a result of the evolving genome (Glynn et al., 1998; Larkin et al., 2006; Threlfall et al., 1994; Zhao et al., 2013). In a study by Zhao et al (2013), when comparing the genetic differences between DT 104 strains to non-DT 104 strains, researchers found there were 4 insertions (ST 104, ST104B, ST64B and SGI-1) that were found in only the DT 104 strains. All of the DT 104 strains contained insertions ST 104, ST104B and ST64B (Zhao et al., 2013); these insertions have been noted in DT 104 strains previously (Hermans et al., 2006). The SGI-1 (*Salmonella* genomic island 1) component, varying in size and present in only some of the DT 104 strains in the study by Zhao et al (2013), has been reported to be responsible for the antimicrobial resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Boyd et al., 2001; Hermans et al., 2006; Hermans et al., 2005; Mulvey et al., 2006; Zhao et al., 2013). *SGI-1* contains five genes, *aadA2, floR, pse-1, sull* and *tet(G)*, which are responsible for the penta-resistance in DT104 (Boyd et al., 2001; Hermans et al., 2006). Serotypes containing the 43-kb *SGI-1* insertion may be more virulent and can easily disseminate (Mulvey et al., 2006). Research has also suggested that the SGI-1 component through horizontal gene transfer is also responsible for ACSSuT resistance in *S. Typhimurium* DT 120 and *S. Agona* serotypes as well (Boyd et al., 2001). Furthermore, Zhao et al (2013) also found one strain with a 144kb plasmid instead of the *SGI-1* insertion that was penta-resistant. This plasmid insertion may be responsible for the evolutionary development of antimicrobial resistance (Zhao et al., 2013).

**1.3.5 Other Resistant *Salmonella* Serotypes**

Although DT 104 is one of the most virulent multi-drug resistant serotypes, there is rapid resistance building in various other *Salmonella* strains. Boyd et al (2001) found the *SGI-1* gene
and ACSSuT resistance in S. Typhimurium DT 120 and S. Agona serotypes. In recent years, monophasic S. Typhimurium has also been emerging (Rabsch et al., 2013). Research has shown a rapid increase in resistance of S. Choleraesuis to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole in Taiwan (Chiu et al., 2004; Chiu et al., 2002; Chu et al., 2001). Research conducted on 60 Alberta swine-finishing farms found S. Derby as the most resistant serotype (Rajić et al., 2004). Other resistant serotypes included S. London, S. Cubana, S. Mbandaka, S. Anatum, S. California, S. I:rough-O:i:1,2, S. I:rough-O:fg:-, S. Orion var 15-34, S. Heidelberg PT29 and S. Ohio (Rajić et al., 2004).

1.3.6 Prevention and Control Measures for AMR

There is a popular belief that to reduce antimicrobial resistance most effectively, you must reduce consumption of antimicrobials in swine and limit the continuous usage of antimicrobial growth promoters (AMGP) in feed (Van den Bogaard et al., 2002). However most of the antibiotics used for growth promotion such as the ionophores have no value in human or veterinary medicine and do not contribute to selective pressure for antimicrobial resistant Salmonella. It appears that multi-drug resistant DT 104 appears to spread from pig farm to pig farm like an infectious disease and is commonly found on farms not using antibiotics, so the simple approach of banning growth promoters is likely to have little or no effect. Alternative solutions are needed. Another means of reducing the resistance would be targeting the plasmids that carry resistance in the pathogens (Van den Bogaard et al., 2002). Along with decreased usage of antimicrobials, pairing that with an effective way to target the plasmids in a “plasmid curing” manner, antimicrobial resistance may gradually decrease (Van den Bogaard et al., 2002).
1.4 Flavophospholipol

Flavophospholipol (bambermycin, flavomycin, moenomycin), discovered in 1955 (Huber, 1979), is a phosphoglycolipid antimicrobial agent produced by *Streptomyces* strains (Bause and Legler, 1982; P Butaye et al., 2000; Pfaller, 2006). The antibiotic predominately functions against Gram-positive bacteria by inhibiting peptidoglycan synthesis in the cell wall (Bause and Legler, 1982; P Butaye et al., 2000; Huber and Nesemann, 1968; Huber, 1979; Pfaller, 2006; Van den Bogaard et al., 2002; Wang et al., 2009). This is facilitated by impairment of the transglycolase activity in the penicillin-binding proteins (PBPs) (Butaye et al., 2000; Huber and Nesemann, 1968; Pfaller, 2006; Van den Bogaard et al., 2002; van Heijenoort et al., 1987; Vanderwel and Ishiguro, 1984). Since flavophospholipol and β-lactam antibiotics affect different PBPs, there is no cross-resistance (Paik et al., 1999; Van den Bogaard et al., 2002).

1.4.1 Effectiveness on Gram-Positive vs. Gram-Negative Bacteria

Flavophospholipol is primarily effective against Gram-positive bacteria (e.g. *Staphylococcus* spp. and *Enterococcus faecalis*) (Butaye et al., 2003; Pfaller, 2006). Gram-positive bacteria (e.g. *Streptococcus pneumonia*) activity is inhibited by flavophospholipol on PBP 2a (Butaye et al., 2003; Paik et al., 1999). However, flavophospholipol is not as highly effective in suppressing Gram-negative bacteria, due to its inability to penetrate the Gram-negative outer membrane (Van den Bogaard et al., 2002). Despite that, studies have shown some activity in flavophospholipol against Gram-negative bacteria like *Pasteurella* spp. and *Brucella* spp. (Huber and Nesemann, 1968; Van den Bogaard et al., 2002). Activity against members of the *Enterobacteriaceae* family, such as *Salmonella* and *E. coli*, have been shown to be slightly
effective (Butaye et al., 2003). PBP 1b, responsible for peptidoglycan bacterial synthesis, in
*E. coli* is suppressed by flavophospholipol (Butaye et al., 2003). While PBP 1a and PBP 3 have
also been found to be sensitive to flavophospholipol in *E. coli* (Butaye et al., 2003; van
Heijenoort et al., 1987).

1.4.2 Use of Flavophospholipol

Due to poor pharmacokinetic and pharmacodynamic features, flavophospholipol does not
meet the minimum requirements to be classified as a therapeutic antimicrobial in human and
veterinary medicine (Pfaller, 2006). The lack of absorption when orally administered, high
concentrations required in parenteral administration, lack of bioavailability and slow excretion
has resulted in the antibiotic not being efficient for treatment purposes (Butaye et al., 2003;
Pfaller, 2006). Attempts to improve the characteristics of the antibiotic have been unsuccessful
(Pfaller, 2006). Without any therapeutic use in human medicine or veterinary medicine,
flavophospholipol develops resistance slowly through chromosomal mutations making it non-
transferable and safe to use in animals (Kissel, 1998a; Pfaller, 2006). Thus, it has been used for
over 30 years primarily as an in-feed AMGP in livestock (Pfaller, 2006; Van den Bogaard et al.,
2002).

1.4.3 Impact on Gut Microflora

Research suggests that flavophospholipol may be capable of reducing *Salmonella*
colonization and shedding in pigs (Dealy and Moeller, 1976; Dorr and Gebreyes, 2009; Van den
Bogaard et al., 2002). Flavophospholipol may have properties that can allow it to alter the gut
microflora in favour of beneficial bacteria inhibiting the colonization of *Salmonella* in animals
(Butaye et al., 2003). This inhibition by flavophospholipol is likely due to competition among pathogenic and beneficial bacteria for either intestinal binding sites or may be due to reduced intestinal pH (Bolder et al., 1999). Through its potential ability to alter the gut microflora, flavophospholipol may also have a positive impact on growth performance and feed conversion efficiency (Butaye et al., 2003; Pfaller, 2006). In an experimental challenge study conducted by Dealy and Moeller (1976), it was found that flavophospholipol was able to reduce the duration and prevalence of Salmonella shedding. Another challenge study found the antibiotic was able to reduce Salmonella colonization in lymph nodes but had no impact on fecal shedding (Letellier et al., 2000). In a study conducted by Van den Bogaard et al. (2002), flavophospholipol helped prevent an increase in the prevalence of resistant E. coli in fecal flora. Whereas, other studies have found no difference (George and Fagerberg, 1984; Smith and Tucker, 1975).

1.4.4 Mechanism against Antimicrobial Resistance

No traces of resistance in plasmids, genes, cross-resistance to other non-phosphorolipid class antibiotics or other resistance determinants in bacterial hosts to flavophospholipol have been found (Butaye et al., 2003; Kissel, 1998a; Pfaller, 2006; Van den Bogaard et al., 2002). Natural resistance to flavophospholipol has been reported in Clostridium perfringens (MIC ≥ 64 µg/mL) and various strains of Enterococcus faecium (MIC > 128 µg/mL) (Aarestrup et al., 1998; Butaye et al., 2003; Pfaller, 2006; Van den Bogaard et al., 2002). High levels of MICs (>128 µg/mL) have been identified in Lactobacillus spp, Bifidobacterium spp, Clostridium spp, wild-type strains of E. coli, Salmonella spp, Klebsiella spp, Pseudomoas spp, and Campylobacter spp (Aarestrup et al., 1998; Devriese and Haeselbrouck, 1996; Dutta and Devries, 1984; Kissel,
Gram-negative bacilli may be intrinsically resistant to flavophospholipol (Van den Bogaard et al., 2002). However, flavophospholipol may have a selective toxicity to Gram-negative bacteria which carry the antimicrobial resistant R-plasmids (Kissel, 1998a, 1998b; Pfaller, 2006; Van den Bogaard et al., 2002; Watanabe et al., 1971). The range of flavophospholipol MIC for E. coli carrying R-plasmid strains was notably less than for E. coli isolates without R-plasmids (Mitsuhashi et al., 1970; Pfaller, 2006; Van den Bogaard et al., 2002; Watanabe et al., 1971). There is a possibility that flavophospholipol may have a particular effect on R-plasmid carrying strains (George and Fagerberg, 1984; Sokol et al., 1973; Van den Bogaard et al., 2002).

Flavophospholipol may be capable of reducing antimicrobial resistance (Dealy and Moeller, 1976; Pfaller, 2006; Van den Bogaard et al., 2002). R-plasmid carrying Salmonella spp. and E. coli strains, with multi-drug resistance, may be sensitive to flavophospholipol activity (Dealy and Moeller, 1976; Kissel, 1998a, 1998b; Pfaller, 2006; Sokol et al., 1973; Van den Bogaard et al., 2002; Watanabe et al., 1971). Flavophospholipol has shown a “plasmid-curing” effect in E. coli and S. Typhimurium resulting in the strains losing their antimicrobial resistant plasmids (George and Fagerberg, 1984; Kissel, 1998a; Pfaller, 2006; Watanabe et al., 1971). Furthermore, the resistant phenotype converted to a susceptible phenotype (George and Fagerberg, 1984; Kissel, 1998a; Pfaller, 2006; Watanabe et al., 1971). Flavophospholipol may instead be inhibiting the growth of bacteria that contain R-plasmids (George and Fagerberg,
1984; Pfaller, 2006; Sokol et al., 1973). Regardless of the mechanism, flavophospholipol has been reported to reduce antimicrobial resistance (Pfaller, 2006).

1.5 Summary

The emergence of multi-drug resistant non-typhoidal Salmonella in swine is an important concern to food safety and public health. Studies show promising yet conflicting results on the impact of flavophospholipol on Salmonella colonization and shedding (Pfaller, 2006; Van den Bogaard et al., 2002). Research also indicates that flavophospholipol can potentially reduce antimicrobial resistance (Pfaller, 2006; Van den Bogaard et al., 2002). Furthermore, studies suggest a positive impact on growth performance and feed conversion efficiency (Butaye et al., 2003; Pfaller, 2006); this could be an economical incentive for swine producers to use the antibiotic.

The majority of the studies on flavophospholipol for control of Salmonella in swine have been experimental challenge trials. Further research is needed to investigate the ability of flavophospholipol in reducing Salmonella colonization/shedding and antimicrobial resistance in naturally infected pigs. Understanding the effectiveness of flavophospholipol as an on-farm intervention for Salmonella may help reduce farm-to-fork transmission. This will help promote food safety nationally and globally. Furthermore, exploring the trends of Salmonella, serotypes, antibodies, and antimicrobial resistance in swine from grower-finisher to slaughter can increase our knowledge and help to lead to better food safety and public health strategies.
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CHAPTER 2: A CLINICAL TRIAL INVESTIGATING THE IMPACT OF IN-FEED FLAVOPHOSPHOLIPOL ON SALMONELLA SHEDDING AND ANTIMICROBIAL RESISTANCE IN PIGS

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2.1 Abstract

A clinical trial was conducted to assess the effectiveness of in-feed flavophospholipol in reducing Salmonella shedding and antimicrobial resistance associated with Salmonella and generic E. coli while improving growth performance in naturally-infected grower-finisher pigs. Pigs were obtained from a farm with a history of salmonellosis and were housed at a research facility. Over the span of 10 weeks the pigs either received a feed containing 4 ppm of flavophospholipol (treatment, n=25) or a non-medicated feed (control, n=20). Weekly fecal samples were collected and cultured for Salmonella and generic E. coli. A subset of Salmonella and E. coli isolates were tested for antimicrobial susceptibility. Findings revealed that the prevalence of pigs shedding Salmonella and the pattern of antimicrobial resistance associated with Salmonella was not different between treatment and control groups (P>0.05). However, flavophospholipol treatment was found to have a protective effect on antimicrobial resistance associated with E. coli isolates (P>0.05).

2.2 Introduction

Non-typhoidal Salmonella spp are commonly recovered in the feces of swine but are rarely found to cause clinical disease (1). Salmonella are highly prevalent on Ontario swine farms (2,3). The most common serotype recovered from Ontario pigs is Salmonella Typhimurium DT104 (2), a multi-drug resistant serotype that is a common human pathogen (4).
Salmonella are difficult to control and furthermore there is little financial incentive for producers to institute costly measures to reduce Salmonella because the affect of these pathogens on pig performance is subtle and under-estimated. However, flavophospholipol (bambermycin, flavomcyin, moenomycin), an inexpensive antibiotic, may be an attractive method for swine producers to reduce on-farm Salmonella and the associated antimicrobial resistance in pigs. Flavophospholipol may provide an improvement in feed efficiency and growth rate and thus provide a financial benefit in addition to the reduction in Salmonella for swine producers.

Flavophospholipol is an antibiotic without any therapeutic applications in humans or veterinary medicine due to its poor pharmacokinetic and pharmacodynamic features, for example it is not absorbed from the intestinal tract when given orally and it’s antimicrobial activity is limited to Gram-positive bacteria (5,6). Resistance to this phosphoglycolipid antimicrobial agent among bacterial populations is slowly developed through mutations in the bacterial chromosome and is non-transferable (5,7). Flavophospholipol may alter the microflora in favour of beneficial bacteria inhibiting the colonization of Salmonella in animals (8). The inhibition by flavophospholipol is likely due to competition among pathogenic and beneficial bacteria for either intestinal binding sites or maybe due to reduced intestinal pH (8). Furthermore, flavophospholipol by inhibiting R-plasmid carrying bacteria in a process referred to as a “plasmid-curing effect” may reduce antimicrobial resistance (9–12). The objective of this study was to evaluate whether the in-feed use of flavophospholipol can reduce Salmonella shedding and antimicrobial resistance associated with Salmonella and generic E. coli.
2.3 Materials and Methods

The research trial was approved by the animal care committee of the University of Guelph, in accordance with the guidelines set forward by the Canadian Council of Animal Care.

Pigs and treatment groups

Forty-five pigs at ten weeks of age were obtained from a commercial farm where Salmonella shedding in pigs was known to be prevalent based on previous diagnostic work. The pigs were transported to the Ponsonby General Animal Facility (University of Guelph) where they were randomly assigned to 1 of 9 pens (5 pigs/pen) and ear tagged. The treatment group consisted of 5 pens (n=25 pigs) and these pigs were provided with a diet containing 4 ppm of flavophospholipol (Flavomycin® Huvepharma, distributed by Bio Agri Mix, Mitchell ON). The control group (n=20 pigs) housed in 4 pens received an identical diet but without the medication. In order to accommodate the growing pigs, at the beginning of Week 8 of the trial, 2-3 pigs were removed from each pen and placed in the corresponding pen in a second, similar room in the research facility. Pigs that had been receiving the treatment ration continued to receive this feed and likewise pigs moved from control pens to the new room continued to receive the control ration. This movement of pigs resulted in a total of 18 pens (10 treatment, 8 control) from Week 8 to Week 11 of the trial. Boot covers and gloves were changed to prevent any transmission of disease between rooms and treatment groups.

Sample collection

Over the 10-week period (Week 1 to Week 11), weekly fecal samples or rectal swabs were collected from the 45 pigs. Samples were collected in stomacher bags directly from pigs as
they defecated and when pigs failed to defecate during the period of observation, rectal swabs were taken. In addition, pooled-fecal samples found on the floor of each pen were collected weekly. Samples were transported to the laboratory in insulated coolers with ice packs.

*Weight and average daily gain*

Pigs were individually weighed at the beginning of Week 1, 4, 7 and 11 of the trial. Average daily gain was calculated for the first 21 days (Week 1 to Week 4), second 21 days (Week 4 to Week 7), the next 28 days (Week 7 to Week 11) and the total 70 days (Week 1 to Week 11).

*Bacterial culturing*

*Salmonella:* The fecal samples were diluted with tetrathionate broth (TTB) (Becton Dickinson™, USA) at a 1 to 9 ratio. The fecal samples were then homogenized for 30 sec with a Seward Stomacher 400 Circulator (Seward, Norfolk, England) and incubated at 37°C for 18 to 24 h. Rectal swabs were cultured in 9 mL TTB and incubated as homogenized fecal samples. Then, 100 µl of TTB culture was transferred to 9.9 mL of Rappaport-Vassiliadis (RV) (Becton Dickinson™, USA) broth and incubated at 41°C for 18 to 24 h. Next, a loopful (~20 µl) of the RV broth was streaked onto xylose-lysine-tergoitol 4 (XLT4, Remel Thermo Fisher Scientific™, USA) agar plates and incubated at 37°C for 18 to 24 h. *Salmonella* isolates were confirmed by *Salmonella* poly O antisera agglutination test (Becton Dickinson™, USA).

*Generic E. coli:* The fecal samples were added to Buffered Peptone Water (BPW, Thermo Scientific™ Oxoid™, USA) at a 1 to 9 ratio, homogenized for 30 sec with a Seward Stomacher 400 Circulator and incubated at 37°C for 18 to 24 h. A loopful (~20 µl) of the BPW
broth was streaked onto MacConkey Agar (BBL™, Becton Dickinson, USA) plate and incubated at 37°C for 18 to 24 h. Colonies were then streaked on to LB Agar, Miller plate (Fisher BioReagents™, USA) and incubated at 37°C for 18 to 24 h. E. coli isolates were identified using spot indole reagent (Remel Thermo Fisher Scientific™, USA) and Simmons Citrate Agar (BBL™, Becton Dickinson, USA) testing.

*Salmonella* serotyping

A subset of *Salmonella* isolates (n=24), the first (n=11) and last positive *Salmonella* culture (n=13) for 13 pigs (7 treatment and 6 control), were submitted to Biovet Inc (St-Hyacinthe, Quebec, Canada) for molecular serotyping by means of xMAP® *Salmonella* Serotyping Assay Kit. The xMAP *Salmonella* serotyping assay is a microsphere-based, molecular serotyping method that detects genes that express serotype-specific O and H antigens.

*Antimicrobial susceptibility testing*

*Salmonella* isolates recovered from pigs at arrival (before treatment), Week 3 and Week 4 (after treatment) as well as *E. coli* isolates recovered from pigs at arrival (before treatment) and Week 11 (after treatment) was tested for antimicrobial susceptibility. Antimicrobial susceptibility testing was performed by means of a micro-dilution broth using a Sensititre™ NARMS Gram negative plate (Thermo Scientific™, USA). The antimicrobials and MIC included were: amoxicillin/clavulanic acid [AUG] (32/16 mg/L), ampicillin [AMP] (32 mg/L), cefoxitin [FOX] (32 mg/L), ceftriaxone [AXO] (64 mg/L), chloramphenicol [CHL] (32 mg/L), ciprofloxacin [CIP] (4 mg/L), gentamicin [GEN] (16 mg/L), nalidixic acid [NAL] (32 mg/L), streptomycin [STR] (64 mg/L), sulfisoxazole [FIS] (256 mg/L), tetracycline [TET] (32 mg/L), trimethoprim/sulphamethoxazole [SXT] (4/76 mg/L). Briefly, isolates were streaked on to LB
agar and incubated for 24 h at 37°C. The colonies on the LB agar were added to sterile water and adjusted to a 0.5 McFarland Standard (TREK Diagnostic System, USA) using the Sensititre® Nephelometer. Then, 30 µl of the McFarland suspension was added to 11 mL of Mueller-Hinton broth with Tes buffer (TREK Diagnostic System, USA). Once mixed well, using the Sensititre® AutoInoculator, 50 µl of the Mueller-Hinton broth suspension inoculated the wells of the plate. Plates were covered with the adhesive seal and labeled. Upon 18 h incubation at 37°C, plates were read using the Sensititre Autoreader® system. Intermediate MIC breakpoints were classified as susceptible.

**Statistical analysis**

Data were entered into Microsoft Excel for Mac 2011 Version 14.5.5 (Microsoft, Redmond, Washington, USA) and then imported into Stata (Stata/SE 14.1 for Mac; StataCorp, College Station, Texas, USA). Differences in average daily gain and weight gain between the treatment and control groups were modeled separately using multilevel mixed-effects linear regression with pen (common environment) and pig (repeated measures) as a random effect. To control for the addition of a second room in Week 8 to Week 11, room was held as a fixed effect. A multilevel mixed-effects logistic regression model with pen and pig as a random effect and room as a fixed effect was used to compare the prevalence of *Salmonella* shedding among pigs in treatment and the control group. A Kaplan-Meier survival function and a log rank test were used to present and evaluate *Salmonella* shedding over the time. The time to event was identified as the last time a pig shed *Salmonella*. A multilevel mixed-effects logistic regression model with pen as a random effect and room as a fixed effect was used to compare antimicrobial resistance in *Salmonella* and *E. coli* isolates recovered from pigs in the treatment and control groups. MIC
values were described using a MIC distribution plot (squashtogram) generated using Microsoft Excel.

2.4 Results

*Salmonella shedding*

Two pigs were euthanized for reasons unrelated to the trial in Week 3 (one from each group). A total of 479 individual fecal samples and 135 pooled-fecal samples were collected. *Salmonella* shedding in pigs and the presence of *Salmonella* in pen samples from Week 1 to Week 11 are shown in Figure 1 and Figure 2, respectively. *Salmonella* was recovered from 27% and 28% of the individual fecal samples from the treatment and the control group, respectively. *Salmonella* was recovered from 36 (80%) pigs at arrival at the research facility (Week 1). Over the 10-week period during which treatment pigs received feed containing 4 ppm of flavophospholipol, the presence of *Salmonella* cultured from fecal samples collected from pens or pigs was similar between treatment and control pigs receiving a non-medicated feed ($P > 0.05$).

The Kaplan-Meier survival function revealed 50% survival probability (pigs stop shedding *Salmonella*) at Week 3 and Week 4 for the treatment and control groups, respectively (Fig 3). All pigs in the control group stopped shedding *Salmonella* by Week 10, while the treatment group stopped shedding at Week 11. The log rank test revealed no significant difference was detected between the survival times in the treatment and control groups ($P > 0.05$).
**Growth performance**

The body weight and average daily gains during different periods of the trial are shown in Table 1 and Table 2, respectively. Pigs who received flavophospholipol did not experience different weight gain ($P>0.05$) or different average daily gain ($P>0.05$) in comparison to the control pigs.

**Salmonella serotypes**

Twenty-four isolates recovered from 13 pigs at different time were serotyped. In total, pigs shed 8 different serotypes over the study period including *S. Typhimurium* (25%), *S. Livingstone* (25%), *S. Senftenberg* (21%), *S. I:Rough-O* (8%), *S. Montevideo* (8%), *S. Benfica* (4%), *S. Amsterdam* (4%), and *S. Infantis* (4%). Of the 11 pigs with first and last isolates, 91% (10) of those were reinfected with a different serotype. Pigs were initially colonized by *S. Typhimurium* (36%), *S. Senftenberg* (36%), *S. Benfica* (9%), *S. Amsterdam* (9%) and *S. I:Rough-O* (9%); however, reinfection with *S. Livingstone* (45%) was the most common followed by *S. Typhimurium* (18%), *S. Montevideo* (18%), *S. Senftenberg* (9%), and *S. I:Rough-O* (9%).

**Antimicrobial resistance**

Minimum inhibitory concentration (MIC) for both *Salmonella* and *E. coli* are shown in the distribution plots (Table 5, 6, 7). Overall, *Salmonella* isolates demonstrated resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline (Table 5 & 6). The antimicrobial resistance profile in *Salmonella* isolates isolated from pigs in control and treatment group at Week 1, 3 and 4 are shown in Table 3. The most prevalent resistance pattern associated
with *Salmonella* in Week 1 is “S” in both the treatment (65%) and control (75%) groups. In Week 3 and 4, “ASSuT” (67%) and “SSuT” (40%) are common in the treatment group while “ASSuT” (Week 3=53%; Week 4=43%) is the most prevalent in the controls (Table 3). *Salmonella* pentaresistant “ACSSuT” was also observed in *Salmonella* isolates (Table 3). There was no significant difference in antimicrobial resistance associated with *Salmonella* isolates recovered from pigs in the treatment and control group (*P*>0.05).

*E. coli* isolated were resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulphamethoxazole (Table 7). Antimicrobial resistance patterns in *E. coli* isolates are shown in Table 4. The most prevalent resistance patterns in *E. coli* isolates in Week 1 are “T” (28%) and “ST” (28%), while “AT” (17%) and “ACSSuT” (17%) are most common in Week 11 in the treatment group. Resistance patterns “ACSSuT” (29%) and “SSuT” (14%) were most common in the control group in Week 1, but during Week 11 “ACSSuTTs” (36%) and “ASSuTTs” (14%) was commonly recovered. *E. coli* isolates recovered from the treatment group were found to have a reduced antimicrobial resistance in ampicillin (OR=0.22, 95% CI [0.06, 0.83], *P*=0.03), chloramphenicol (OR=0.25, 95% CI [0.08, 0.76], *P*=0.01), and trimethoprim/sulphamethoxazole (OR=0.10, 95% CI [0.02, 0.52] *P*=0.01) in comparison to the control group.

### 2.5 Discussion

One objective of this study was to investigate the impact of in-feed flavophospholipol on *Salmonella* shedding in pigs. Our findings indicate that in-feed flavophospholipol was ineffective in reducing *Salmonella* shedding in naturally-infected pigs. Some experimental challenge studies
have found flavophospholipol to be effective in reducing *Salmonella* shedding in swine and broilers (8,13). Research conducted by Dealy and Moeller (13) found flavophospholipol to significantly reduce the duration and prevalence of *Salmonella* shedding in *Salmonella Typhimurium* challenged pigs, while reducing the number of isolates resistant to ampicillin, streptomycin, triple sulfa and tetracycline. Bolder et al. (8) found flavophospholipol to decrease the rate and magnitude of *Salmonella* in broilers. In contrast, other experimental challenge studies conducted in chickens and pigs with *Salmonella Typhimurium* found the antibiotic to not be effective in reducing *Salmonella* (14,15).

Unlike the challenge studies where animals were experimentally infected with *Salmonella* and fed flavophospholipol for a short period, the current study used naturally-infected pigs and followed these pigs over 10 weeks which was more representative of the farm situation. One difference between controlled challenge studies and the present study was that this population of pigs was infected with multiple serotypes, the most prevalent being *Salmonella Typhimurium* followed by *Salmonella Livingstone*. This makes it difficult to estimate the effectiveness of flavophospholipol on *Salmonella* shedding because multiple serotypes resulted in reinfections and renewed shedding. Furthermore, the pathogen may have already established a permanent infection in the pigs during the nursery stage. If a high degree of *Salmonella* colonization occurred in the gut flora prior to the pigs receiving treatment, this may have enabled flavophospholipol from having an impact on the beneficial bacteria. If these same pigs were provided with the treatment diet at an earlier stage (i.e. after weaning) when the pigs were younger and most susceptible, a more favourable outcome may have occurred. Future studies to examine the benefits of administering flavophospholipol, as a preventive measure, before pigs
are infected with *Salmonella* needs to be investigated.

The second objective was to study the impact of in-feed flavophospholipol on antimicrobial resistance in *Salmonella*. While other studies found flavophospholipol to be capable of reducing antimicrobial resistance (13,16), the present study found no difference in antimicrobial resistance in *Salmonella* when treated with flavophospholipol. It is possible that looking for a difference after 3 and 4 weeks of treatment with flavophospholipol may not be an adequate measure. An earlier study by Dorr and Gebreyes (16) recovered *Salmonella* isolates with significantly less resistance to ampicillin and amoxicillin-clavulanic acid from pigs challenged with *Salmonella* Typhimurium DT104 and DT193 after being treated with flavophospholipol. However, this study (16) compared antimicrobial resistance from treated isolates recovered over 20 weeks to controls whereas Dealy and Moeller (13) compared treated isolates recovered over 7 weeks to controls. The antimicrobial resistance curing properties of flavophospholipol may require a longer duration to be effective.

Challenge studies that found flavophospholipol to significantly reduce antimicrobial resistance colonized pigs with one strain of *Salmonella* (13,16). There is a possibility that flavophospholipol was ineffective in the present study due to the presence of multiple serotypes resulting in reinfections. Antimicrobial susceptibility testing was conducted on isolates from Week 3 and 4 due to the large prevalence of *Salmonella* in pigs. However, majority of the pigs were found reinfected at Week 3 and 4 with a different serotype from Week 1. Although the present study may have been representative of the farm situation, it is difficult to estimate the effectiveness of flavophospholipol on *Salmonella* shedding and antimicrobial resistance of *Salmonella*. 
The impact of in-feed flavophospholipol on antimicrobial resistance in generic *E. coli* in pigs was also examined. In the present study, antimicrobial resistance in *E. coli* isolated from flavophospholipol treated pigs had less resistance than isolates from control pigs. If antimicrobial susceptibility testing in *Salmonella* isolates, like *E. coli*, was conducted at Week 1 and Week 11 a more favourable outcome may have been witnessed. The research findings supports earlier work by van den Bogaard et al. (12) that found flavophospholipol to effectively suppress multidrug resistant *E. coli* in challenged pigs. This study used a dosage of 9 ppm of flavophospholipol (12). However, van den Bogaard et al. (12) found a decrease in the mean degree of resistance in oxytetracycline whereas the present study found a decrease in ampicillin, chloramphenicol and trimethoprim/sulphamethoxazole. Based on these findings, the standard dosage of (4 ppm) flavophospholipol may be sufficient in reducing antimicrobial resistance in generic *E. coli* isolates.

Flavophospholipol, suggested to have a positive impact on growth performance, is currently used as a growth-promoting antibiotic in various livestock (5,6). Although not an objective of the study due to the small numbers of animals used in the study, growth and feed efficiency was measured. As one would expect from the small sample size the growth rate in the treatment and control pigs in this present study was similar. Although flavophospholipol is licensed for use for growth promotion in poultry in Canada, a previous study by Bolder et al (8) found no difference between the flavophospholipol treatment and control groups when using *Salmonella*-challenged broilers. Since this was not the primary objective of this study, the statistical power to detect an effect was predictably low.
In conclusion, naturally-infected pigs subsequently given flavophospholipol at 4 ppm in feed continued to shed *Salmonella* for a similar length of time compared to non-medicated pigs. The antimicrobial resistance pattern of *Salmonella* isolates was not improved by feeding pigs a diet containing 4 ppm of flavophospholipol after 2 or 3 weeks. However, *E. coli* isolates showed improved resistance over ten weeks. Therefore, flavophospholipol does not appear to be an effective strategy in reducing *Salmonella* prevalence or antimicrobial resistance in a population of pigs infected with multiples strains of *Salmonella*. Future studies are needed to investigate the preventive effect of flavophospholipol if administered at earlier stage of production (during the nursery stage) before pigs become infected with *Salmonella*.

### 2.6 References

7. Kissel A. Determination of the in-vitro susceptibility of selected bacterial strains carrying resistance plasmids to the feed additives salinomycin–sodium (SAL) and flavophospholipol (FPL) and to various active substances used for therapeutic purposes—


Table 2.1: The mean weight (kg) with standard deviation and range for Week 1, Week 4, Week 7 and Week 11 in the flavophospholipol treatment group and the control group

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Mean (SD)</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>22 (2.26)</td>
<td>19.6, 27.3</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>22 (2.50)</td>
<td>18.3, 28.1</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>36 (3.31)</td>
<td>31.2, 42.3</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>38 (4.19)</td>
<td>30.3, 45.6</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>57 (5.36)</td>
<td>45.3, 65.5</td>
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<td>Treatment</td>
<td>57 (6.22)</td>
<td>44.4, 66.3</td>
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<td>Control</td>
<td>91 (9.10)</td>
<td>70.0, 104.1</td>
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<tr>
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<td>Treatment</td>
<td>90 (10.60)</td>
<td>64.5, 104.7</td>
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</table>
Table 2.2: The mean average daily gain (kg/day) with standard deviation and range for Week 1 to 4, Week 4 to 7, Week 7 to Week 11 and Week 1 to Week 11 in the flavophospholipol treatment group and the control group

<table>
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<th>Week</th>
<th>Group</th>
<th>Mean (SD)</th>
<th>Range</th>
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</thead>
<tbody>
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<td>1-4</td>
<td>Control</td>
<td>0.66 (0.10)</td>
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<td>Treatment</td>
<td>0.74 (0.14)</td>
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<td>Treatment</td>
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<td>7-11</td>
<td>Control</td>
<td>1.20 (0.20)</td>
<td>0.87, 1.56</td>
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<td>Treatment</td>
<td>1.15 (0.28)</td>
<td>0.07, 1.45</td>
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<tr>
<td>1-11</td>
<td>Control</td>
<td>0.98 (0.11)</td>
<td>0.72, 1.16</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.96 (0.14)</td>
<td>0.61, 1.21</td>
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</table>
Table 2.3: *Salmonella* isolate resistance patterns in treatment (flavophospholipol) and control groups at Week 1 (before treatment), at Week 3 and Week 4 (during treatment)

<table>
<thead>
<tr>
<th>AMR pattern</th>
<th>Week 1 (%)</th>
<th>Week 3 (%)</th>
<th>Week 4 (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Treatment (n=17)</td>
<td>Control (n=16)</td>
<td>Treatment (n=15)</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S</td>
<td>65</td>
<td>75</td>
<td>7</td>
</tr>
<tr>
<td>AT</td>
<td>6</td>
<td>0</td>
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</tr>
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<td>6</td>
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<tr>
<td>SSu</td>
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<tr>
<td>SSuT</td>
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<td>ASSuT</td>
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<tr>
<td>ACSSuT</td>
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<td>6</td>
<td>7</td>
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*ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), tetracycline (T)
Table 2.4: *E. coli* resistance patterns in treatment (flavophospholipol) and control groups at Week 1 (before treatment) and at Week 11 (after treatment)

<table>
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<tr>
<th>AMR pattern</th>
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<th>Week 11 (%)</th>
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<td>Treatment (n=18)</td>
<td>Control (n=14)</td>
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<tr>
<td>T</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>ST</td>
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<td>0</td>
</tr>
<tr>
<td>AT</td>
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<td>7</td>
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<tr>
<td>AST</td>
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<tr>
<td>SSuT</td>
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<tr>
<td>ASSuT</td>
<td>11</td>
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<td>CSSuT</td>
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<td>ACST</td>
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<td>AGSSuT</td>
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<td>ACSSuT</td>
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<td>29</td>
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<tr>
<td>AcASuT</td>
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<tr>
<td>CNSSuT</td>
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*amoxicillin/clavulanic (Ac), ampicillin (A), cefoxitin (Ce), chloramphenicol (C), ciprofloxacin (Ci), gentamicin (G), nalidixic (N), streptomycin (S), sulfonamides (Su), tetracycline (T), trimethoprim/sulphamethoxazole (Ts)
Table 2.5: Comparison of resistance and MIC distribution (squa shotgun) for *Salmonella* isolates recovered from flavophospholipol treated (Tx; n=30) (n=isolates) and control (C;n=30) pigs at Week 1 and Week 3

<table>
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<th>0.06</th>
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<th>0.5</th>
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<tr>
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<tr>
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Areas with white backgrounds indicate the range of dilutions tested for each antimicrobial. Shaded areas fall outside the range of tested concentrations. Numbers in the right-side shaded areas indicate the percentage of isolates with undetermined MICs known to be greater than the highest concentrations measured.

*a* AUG, amoxicillin/clavulanic acid; AMP, ampicillin; FOX, cefoxitin; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline; SXT, trimethoprim/sulphamethoxazole

*b* MIC represents the first antibiotic (of two)
Table 2.6: Comparison of resistance and MIC distribution (squaresogram) for *Salmonella* isolates recovered from flavophospholipol treated (Tx; n=10) and control (C;n=14) pigs at Week 1 and Week 4

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Table 2.7: Comparison of resistance and MIC distribution (squares) for *E. coli* isolates recovered from flavophospholipol treated (Tx; n=36) and control (C; n=30) pigs at Week 1 and Week 11.
Figure 2.1: *Salmonella* shedding in pigs in the treatment (flavophospholipol) and control group from Week 1 to Week 11
Figure 2.2: *Salmonella* status in pens in the treatment (flavophospholipol) and control group from Week 1 to Week 11
**Figure 2.3:** Survival probability (time to event: when pigs stop shedding *Salmonella*) of the treatment (flavophospholipol) group versus the control group from Week 1 to Week 11.
CHAPTER 3: TIME COURSE OF SALMONELLA SHEDDING AND ANTIBODY RESPONSE TO SALMONELLA IN NATURALLY-INFECTED PIGS THROUGH THE GROWER-FINISHER STAGE UNTIL SLAUGHTER

3.1 Abstract

A longitudinal trial was conducted to determine the course of Salmonella shedding and antibody response in naturally-infected grower-finisher pigs. Ten-week-old pigs (n=45) were transferred from a farm with a history of salmonellosis and housed at a research facility. Weekly fecal samples (Week 1-11) as well as tissue samples were collected at slaughter and cultured for Salmonella. Serum samples were tested for presence of Salmonella antibody by ELISA. Data were analyzed using a multilevel mixed-effects logistic regression model. Of the 45 pigs, 41 (91%) shed Salmonella ≤4 times and 4 (9%) pigs ≥5 times. The estimated median of Salmonella shedding duration was 3-4 weeks but some pigs shed Salmonella for up to 8 weeks. Salmonella shedding decreased from 12 to 20 weeks of age (P<0.05). Salmonella isolates (n=89) belonged to C1 (44%), E4 (29%), B1 (19%) and other serogroups (8%). During the study period, 70.3% (n=26/37) of pigs were re-infected with Salmonella belonging to a different serogroup. A subset of isolates (n=29), which were recovered from 18 pigs at different occasions, belonged to serotypes S. Typhimurium (28%), S. Livingstone (21%), S. Infantis (14%), S. Montevideo (7%), S. Benfica (3%), S. Amsterdam (3%), S. Senftenberg (17%), and S. I:Rough-O (7%). Of 11 pigs serotyped for the first and last isolate, 91% (10) of those were reinfected with a different serotype. At slaughter, Salmonella was isolated from 7 pigs of which, 5 (71%) had not been tested positive on weekly fecal checks for at least 7 weeks or longer. Antibody response peaked
4 weeks after the peak of *Salmonella* infection; *Salmonella* shedding reduced as antibody response elevated ($P<0.05$). These findings indicate that pigs may shed *Salmonella* into the mid-point of the grower-finisher stage and may be reinfected with different serotypes. Further, the absence of *Salmonella* detection in fecal samples in the late finisher stage may not be indicative of the carcass being *Salmonella*-free at slaughter.

### 3.2 Introduction

The emergence of multi-antimicrobial resistant *Salmonella* commonly present on swine farms presents a public health and food safety concern. Understanding the dynamics and epidemiology of *Salmonella* infection in the swine population is vital in improving prevention and control of *Salmonella* at the farm level and reducing the “farm-to-fork” transmission of the pathogen. The prevalence of *Salmonella* shedding has been reported to be highest in the nursery stage and as pigs age, through the grower-finisher period, it has been found that there is a decline in *Salmonella* shedding until slaughter (Kranker et al., 2003; Nollet et al., 2005). However, various longitudinal studies have reported variability in *Salmonella* shedding patterns and have detected a higher than expected prevalence of *Salmonella* shedding in the grower-finisher stage (Funk et al., 2001; Wilkins et al., 2010). A great variability also exists from the onset of infection to the time of host antibody response (Nielsen et al., 1995). Furthermore, the distribution of serotypes can greatly impact the duration of *Salmonella* shedding (Funk et al., 2001; Pires et al., 2014) and affect whether *Salmonella* may be recovered from tissue at slaughter or not (Gebreyes et al., 2004).

The objectives of this study were: (i) to investigate the *Salmonella* shedding pattern in naturally-infected pigs through the grower-finisher stage, (ii) to examine the development of
antibody levels in relation to the *Salmonella* shedding pattern, and (iii) to determine the presence of *Salmonella* in tissue and cecal content at slaughter.

### 3.3 Methods and Material

The research trial was approved by the animal care committee of the University of Guelph, in accordance with the guidelines set forward by the Canadian Council of Animal Care.

**Pigs**

A longitudinal trial was conducted for duration of 10 weeks (starting at Week 1 and ending at Week 11). This was followed shortly after by slaughter, due to convenience, at Week 13 and Week 14. Prior to the start of the study, a commercial swine farm in Ontario (Canada) with a history of high *Salmonella* prevalence was identified and visited. *Salmonella* was recovered from gestation sow stalls and nursery pens on this operation. Forty-five naturally-infected 10-week-old feeder pigs weighing approximately 25 kg from this farm were purchased and transported to Ponsonby General Animal Facility (University of Guelph). The pigs were ear tagged for individual identification and randomly assigned to 1 of 9 pens with 5 pigs/pen from Week 1 to Week 7. In order to accommodate the growing pigs, at the beginning of Week 8 of the trial, 2-3 pigs were removed from each pen and placed in the corresponding pen in a second, similar room in the research facility. This resulted in a total of 18 pens from Week 8 to Week 11.

**Sample collection**

Over the first 10 weeks that the pigs were at the research facility weekly, fresh individual-animal fecal samples (5-25 g) or rectal swabs were collected. Blood samples were collected from each pig during Week 1, 4, 7 and 11. Samples were transported to the laboratory in insulated coolers with ice packs. Blood samples were centrifuged and stored at -20 C.
**Slaughter sampling**

Two to three weeks after the last sampling, pigs were sent to slaughter at 22 to 23 weeks of age. Animals were transported in small groups of about 15 animals, and housed individually during the short waiting time (<1h) from arrival until slaughter. At slaughter, tissues samples from the liver, spleen, neck lymph node, ileocecal lymph node, and tonsil as well as the cecal contents were collected from each pig. All samples were transported to the laboratory in insulated coolers with ice packs.

**Salmonella isolation**

The fecal and tissue samples were diluted with tetrathionate broth (TTB) (Becton Dickinson™, USA) at a ratio of 1 to 9. The samples were homogenized for 30 sec (1 min for tissue samples) with a Seward Stomacher 400 Circulator (Seward, Norfolk, England) and incubated at 37°C for 18 to 24 h. Then, 100 µl of TTB broth was transferred to 9.9 mL of Rappaport-Vassiliadis (RV) (Becton Dickinson™, USA) broth and incubated at 41°C for 18 to 24 h. Next, a loopful (~20 µl) of the RV broth was streaked onto xylose-lysine-tergoitol 4 (XLT4, Remel Thermo Fisher Scientific™, USA) selective agar plates and incubated at 37°C for 18 to 24 h. Any suspected *Salmonella* colonies were tested using *Salmonella* poly O antisera agglutination test (Becton Dickinson™, USA).

**Salmonella serogrouping and serotyping**

The *Salmonella* isolates (n=89) from the first and last shedding per pig and the isolates recovered from tissue samples were serogrouped by agglutination slide test using BD antisera (Becton, Dickinson and Company, Franklin Lakes, New Jersey). In addition, a subset of these isolates (n=29) were submitted to Biovet Inc (St-Hyacinthe, Quebec, Canada) for molecular
serotyping by means of xMAP® Salmonella Serotyping Assay Kit. The subset of isolates included the first shedding isolate (n=11) and last shedding (n=13) for 13 pigs and tissue isolates (n=5) from 5 other pigs. The xMAP Salmonella serotyping assay is a microsphere-based, molecular serotyping method that detects genes that express serotype-specific O and H antigens.

**Salmonella antibody detection**

Serum samples were assessed for presence of antibodies using an indirect enzyme-linked immunosorbent assay (ELISA) pigtype® Salmonella Ab kit (QIAGEN Leipzig GmbH, Leipzig, Germany). The coating Salmonella antigens (LPS) on the microtiter test plates were sensitive in detecting antibodies to Salmonella serogroups B, C, D and E (O-antigens 1, 3, 4, 5, 6, 7, 9, 10 and 12). ELISA was performed as described by manufacturer. A pig was identified as seropositive if the adjusted antibody optical density (OD) was greater than 0.3 and seronegative if the adjusted antibody OD was less than 0.3.

**Data analysis**

Data were entered into Microsoft Excel for Mac 2011 Version 14.5.5 (Microsoft, Redmond, Washington, USA) and then imported into Stata (Stata/SE 14.1 for Mac; StataCorp, College Station, Texas, USA). A Kaplan-Meier survival function was used to present and evaluate Salmonella shedding over the time. The time to event was identified as the last time a pig shed Salmonella. A multilevel mixed-effects logistic regression model with pen (common environment) and pig (repeated measurement) was used to analyze the prevalence of Salmonella shedding overtime. To control for the addition of a second room in Week 8 to Week 11, room was held as a fixed effect. In addition, a multilevel mixed-effects logistic regression model with
pen and pig as random effect and room as a fixed effect was used to compare antibody response (seropositivity) with *Salmonella* shedding.

### 3.4 Results

**Salmonella shedding**

Two pigs were euthanized for reasons unrelated to the trial in Week 3. A total of 479 individual fecal samples were collected over the 10-week duration of the trial. All pigs tested positive for *Salmonella* shedding at least once with 89% of pigs testing positive more than once (Fig 1). *Salmonella* could be recovered between 1 to 8 times from fecal samples collected from each pig weekly. Out of the 45 pigs, 41 pigs were positive 4 times or less and 4 pigs tested positive 5 times or more over the 10 week trial (Fig 1). These four pigs were classified as chronic shedders, as they were repeatedly positive throughout the trial. *Salmonella* was recovered from 36 (80%) pigs at the arrival to research facility (Week 1; 10 weeks of age) (Fig 2). The remaining 9 (20%) pigs were positive at 11 or 12 weeks of age (Fig 1). *Salmonella* shedding declined in Week 2 (38%) but increased in Week 3 (91%). From Week 3 onwards, shedding *Salmonella* was shown a decreasing trend. The overall mean prevalence of *Salmonella* shedding was 27% from Week 1 to Week 11.

The Kaplan-Meier survival function revealed 50% survival probability (pigs stop shedding *Salmonella*) was at Week 3 (12 weeks of age) and 80% survival probability was at Week 6 (15 weeks of age) (Fig 3). However, the survival curve shows *Salmonella* shedding until the end of the trial, with 90% survival probability at Week 9 (18 weeks of age); *Salmonella* was recovered from 2 (5%) pigs at Week 11 (Fig 3). Using the survival analysis data of when pigs started (first culture positive) and stopped shedding *Salmonella* (last culture positive), the
duration of *Salmonella* shedding was estimated. The estimations were based on the assumptions that a pig was shedding for a week prior to the first isolation and was shedding a week after the last isolation (Kranker et al., 2003). Based on these assumptions, the median time of shedding was roughly 3 or 4 weeks. The two longest durations of *Salmonella* shedding was estimated to be 3 or 4 weeks (17 pigs; 37.8%) and 4 or 5 weeks (10 pigs; 22.2%). The range of *Salmonella* shedding was from 1 to 12 weeks, with two pigs shedding *Salmonella* for the entire study.

When analyzing *Salmonella* shedding as pigs aged using a multilevel mixed-effects logistic regression model, the addition of the second room at Week 8 was found to have no impact. Modeling age as ordinal data, analysis revealed that as pigs aged from 11 weeks of age to 12 weeks of age (Week 2 to Week 3) the odds of *Salmonella* shedding was 18.5 times (95% CI [2.85, 119]) greater in comparison to pigs as they aged from 10 weeks of age to 11 weeks of age (Week 1 to Week 2; referent) \( (P=0.002) \). Whereas, from 12 weeks of age to 20 weeks of age (Week 3 to Week 11) there was a significant decrease in *Salmonella* shedding in comparison to pigs at 11 weeks of age to 12 weeks of age \( (\text{OR}=0.002, 95\% \text{ CI} [0.00, 0.01], P=0.001) \). Further, the pigs deemed less likely to shed *Salmonella* as getting older \( (P=0.05) \).

*Salmonella antibody*

The mean level of adjusted optical density (OD) in pigs is shown in Figure 2. As the mean level of OD (at Week 1, Week 4, Week 7 and Week 11) increased, the prevalence of *Salmonella* shedding (Week 1 to Week 11) decreased (Fig 2). Similarly, the predictive probability of *Salmonella* shedding was decreasing as the level of antibodies increased (Fig 4). Multilevel mixed effect analysis revealed *Salmonella*-shedder pigs are less likely to be tested
seropositive by ELISA than non-shedder pigs while controlling for clustering at the pen level and pig level (OR= 0.38, 95% CI [0.15, 0.98], \( P=0.04 \)). Furthermore, the results showed the *Salmonella* seroprevalence (16 weeks of age) to peak 4 weeks (28 days) after the peak in bacteriology culturing (12 weeks of age).

**Presence of *Salmonella* in tissue samples**

At slaughter, 16% (7/43) of pigs tested positive for *Salmonella* in at least one or more tissue or cecal content culture; one pig tested positive for two tissue samples. The mean prevalence of *Salmonella* in tested slaughter samples was 3.15% (8/254; samples tested). *Salmonella* was recovered only from one ileocecal lymph node, one neck lymph node, one spleen, one tonsils, and one liver but from three cecal contents. The majority of pigs (71% (5/7)) harboring *Salmonella* at slaughter had tested negative on fecal culture for 7 weeks or longer prior to slaughter (Fig 1). When assessing the antibody levels in pigs that were *Salmonella* positive at slaughter, two (29%) pigs were found seronegative at week 11 (last *Salmonella* culture positive at week 3) and the other pig during the entire trial (last *Salmonella* culture positive at week 4).

**Salmonella serogroups and serotypes**

Eighty-nine isolates recovered from 37 pigs at different occasion belonged to C1 (44%), E4 (29%), B1 (19%) and other serogroups (8%). Furthermore, 70.3% (26/37) of pigs were reinfected with *Salmonella* belonging to a different serogroup during the study period; E4 serogroup to C1 (37%; 13/37) and B (11%; 4/37) serogroup was the most frequent change. The majority of these changes were seen in Week 3 and Week 4. In addition, pigs shedding serogroup C1 did not change serogroups (14%; 4/37). Twenty-nine isolates recovered from 18 pigs at different time were serotyped. In total, pigs shed 8 different serotypes over the study period.
including *S.* Typhimurium (28%), *S.* Livingstone (21%), *S.* Infantis (14%), *S.* Montevideo (7%), *S.* Benfica (3%), *S.* Amsterdam (3%), *S.* Senftenberg (17%), and *S.* I:Rough-O (7%).

*Salmonella* isolates recovered from tissue samples were Infantis and Typhimurium. Of 11 pigs serotyped for first and last isolate, 91% (10) of those were reinfe-
ccted with a different serotype. Pigs were initially colonized by *S.* Typhimurium (36%), *S.* Senftenberg (36%), *S.* Benfica (9%), *S.* Amsterdam (9%) and *S.* I:Rough-O (9%); however, reinfection with *S.* Livingstone (45%) was the most common followed by *S.* Typhimurium (18%), *S.* Montevideo (18%), *S.* Senftenberg (9%), and *S.* I:Rough-O (9%).

### 3.5 Discussion

This population of pigs had a high prevalence of *Salmonella* shedding and although they appeared to be clinically healthy pigs that came from a single source, they were infected with multiple serotypes. The general pattern of *Salmonella* shedding witnessed during this trial was a high prevalence of *Salmonella* during the early grower phase and a decline in shedding up until slaughter. The prevalence of *Salmonella* shedding in the pigs entering the trial was very high and may have been a result of the stress of transport (Nollet et al., 2005; Pires et al., 2012). Research suggests that stress, in particular the increase in cortisol, experienced during transport intensifies the colonization of *Salmonella* (Verbrugghe et al., 2011). Another possibility is that the pigs had already been colonized by *Salmonella* during the nursery stage (Pires et al., 2012).

The variability in *Salmonella* prevalence and shedding pattern during the early grower phase may be due to reinfection and renewed *Salmonella* shedding caused by the presence of multiple serotypes. Although pigs are deemed less likely to shed *Salmonella* as getting older, findings indicated an increased prevalence of *Salmonella* shedding from 11 weeks of age to 12
weeks of age. This was a result of pigs experiencing reinfection with different serotypes of *Salmonella* during this period of time. Furthermore, research suggests that transmission of *Salmonella* may be dependent on serotypes (Van Winsen et al., 2001). In particular, serotype *S. Livingstone*, most commonly found to cause reinfection in pigs in the present study, has been found to have rapid transmission (Van Winsen et al., 2001). Earlier studies have reported multiple serotypes in one pig and amongst a group of pigs (Funk et al., 2001; Gebreyes et al., 2004; Rajić et al., 2005), similar to the findings from the present study. Furthermore, majority of serotypes recovered from this population of pigs have been previously reported in Ontario swine farms (Farzan et al., 2008; Farzan et al., 2008b).

Previous research has found that pigs with the onset of infection at 10 weeks of age had a longer duration of infection suggesting that younger pigs may be more susceptible to *Salmonella* as it may be easier to establish permanent infections (A. F. Pires et al., 2012). In the present trial, majority of pigs were infected at 10 weeks of age and were found to shed *Salmonella* for up 8 weeks. Furthermore, the estimated median time of *Salmonella* shedding in pigs was longer than results reported of two weeks (range 2-16 weeks) (Pires et al., 2012) and closer to findings of 2-4 weeks (18 or 26 days) (range 1-14.4 weeks) described by Kranker et al (2003). Overtime, as the pigs progressed through the grower-finisher period, there was an overall reduction in *Salmonella* shedding. Low levels of *Salmonella* before slaughter found in the present study have also been previously reported in other longitudinal studies (Belœil et al., 2004; Funk et al., 2001; Kranker et al., 2003; Nollet et al., 2005).

As *Salmonella* shedding reduced over the grower-finisher period, the level of antibodies increased in pigs in the present study. Previous findings from a longitudinal study, conducted on
3 farrow-to-finish swine herds in 2 cohorts with a S. Typhimurium infection, reported a peak in seroprevalence in mid-finishing stage at 17 weeks of age with the antibody response approximately 60 days after the peak in Salmonella shedding (Kranker et al., 2003). A similar peak in seroprevalence was noted in the present study, however the antibody response was found to occur much sooner after the peak in Salmonella shedding. Whereas a longitudinal study using 256 farrow-to-finish pigs found a delayed onset of Salmonella seropositivity until the last third of the finishing phase with Salmonella shedding predominantly in the first half of the grower-finisher stage (Belœil et al., 2003). Findings from an experimental challenge study by Nielsen et al (1995) using S. Typhimurium and S. Infantis found pigs to become seropositive at day 7 and peak in seroprevalence at 30 days post inoculation. Furthermore, this study also found variability in the time of seropositivity, the strength of OD and the persistence of the antibody response amongst pigs (Nielsen et al., 1995). However, due to the small sample size in the present study, the antibody response findings cannot be extrapolated to longitudinal studies with a broad population. Also, the results from the present study cannot be compared to a challenge studies because the pigs in the present study were naturally infected with multiple serotypes.

Following the low levels of Salmonella in the late finisher stage in the present study, S. Typhimurium and S. Infantis recovered at slaughter have been reported in tissue and cecal content at slaughter in earlier studies (Cote et al., 2004; Gebreyes et al., 2004; Scherer et al., 2008). Research suggests pigs infected with S. Typhimurium are asymptomatic carriers that typically colonize Salmonella in their tissues (tonsils, guts, gut-associated lymph nodes) for weeks up to months (Woods et al., 1989) with low to no shedding of Salmonella prior to slaughter (Verbrugghe et al., 2012). However, two of the chronic shedders in this trial were
infected with *S. Typhimurium* and the strain was recovered from both fecal samples toward
the end of the trial and from tissues at slaughter. Furthermore, *S. Livingstone* that was highly
recovered from pigs in this study could not be isolated at slaughter. Further research assessing
*Salmonella* serotypes can be helpful in understanding which strains cause prolonged *Salmonella*
shedding and are more prone to colonizing in tissue.

The present study found that the majority of pigs *Salmonella*-positive at slaughter were
seropositive although they were found *Salmonella* negative using bacterial culture. Similarly,
Nielsen et al (1995) found *S. Typhimurium* challenged pigs that had been bacterial culture
negative for 65 to 106 days and positive at slaughter were seropositive. *S. Infantis* was also
commonly recovered at slaughter from intermittent carriers that had be *Salmonella* culture
negative for over 7 weeks in the present study. The serological testing methods may be able to
identify pigs that are colonized by *Salmonella* when bacterial culture is not able to identify these
asymptomatic *Salmonella* carrier pigs (Lo Fo Wong et al., 2004). Furthermore, a high level of
seropositivity in the grower-finisher phase may be indicative of the presence of *Salmonella* at
various stages in production (Lo Fo Wong et al., 2004).

The present study, with a population of pigs infected with multiple serotypes, is a good
representation of a farm highly infected with *Salmonella*. Findings from the present study and
previously published studies reveal variability in *Salmonella* shedding, duration and antibody
response to *Salmonella* and this may be due to the differences in sampling methods, virulence of
different serotypes, genetics of pigs and husbandry practices (Nielsen et al., 1995; Rajić et al.,
2005). Many farm prevalence studies have used fecal culture of pigs close to market weight and
by sampling these older animals the true on-farm prevalence may appear lower than it actually is
if the shedding pattern is similar to the present study. In the present study, pigs were weekly tested by *Salmonella* fecal culturing and by multiple serological testing. Although this increased the probability of *Salmonella* detection (both shedding and antibody response), the study is limited to a small population of pigs from a single source and the findings may not being extrapolated to a broad population or compared to prevalence studies. Furthermore, the present study cannot be compared to many challenge studies that assess the prevalence of *Salmonella* shedding and antibody response to *Salmonella* in pigs colonized by one serotype.

In conclusion, *Salmonella* shedding in naturally-infected pigs was found to peak in the early grower stage and decline in the mid-finishing stage. From the peak of *Salmonella* infection in pigs, antibody response was delayed 4 weeks with the peak level of antibodies occurring in mid-finishing stage. At slaughter, *Salmonella* was recovered from pigs that were *Salmonella* negative based on fecal culture for 7 to 8 weeks prior. Future studies evaluating serological testing to identify asymptomatic *Salmonella*-carrier pigs is important. Furthermore, the presence of multiple serotypes in pigs may result in reinfections and variability in the shedding of *Salmonella*. Further research assessing *Salmonella* serotypes can be helpful in understanding which strains cause prolonged *Salmonella* shedding and are more prone to colonizing in tissue.

### 3.6 References


Verbrugghe, E., V. Vandenbroucke, M. Dhaenens, N. Shearer, J. Goossens, S. De Saeger,… F. Pasmans, 2012: T-2 toxin induced Salmonella Typhimurium intoxication results in decreased Salmonella numbers in the cecum contents of pigs, despite marked effects on Salmonella-host cell interactions. Veterinary Research. 43(1), 22.


**Figure 3.1:** This chart illustrates the pig’s *Salmonella* status (determined by *Salmonella* culturing) from the beginning of the trial at Week 1 (W1) to Week 11 (W11) and at slaughter (SH) based on how many times the pig was positive (counts) by trend of *Salmonella* shedding grouped together.

<table>
<thead>
<tr>
<th>Counts (No. of pigs)</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
<th>W7</th>
<th>W8</th>
<th>W9</th>
<th>W10</th>
<th>W11</th>
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<td>1 (5)</td>
<td>1</td>
<td>3</td>
<td>1*</td>
<td>2</td>
<td>1</td>
<td>8</td>
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<td>2 (13)</td>
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<td>5</td>
<td>4</td>
<td>1</td>
<td>2</td>
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</table>

Grey squares present *Salmonella* positive and white squares represent *Salmonella* negative.

* censored
**Figure 3.2:** The prevalence of *Salmonella* shedding from Week 1 (W1) to Week 11 (W11) versus the mean level of serum adjusted optical density (OD) obtained by ELISA in pigs at Week 1, 4, 7 and 11 (adjusted optical density represents the amount of antibody against *Salmonella* in serum samples)
**Figure 3.3:** Kaplan-Meier Survival Curve illustrates the survival probability of *Salmonella* shedding over 10 weeks with the time to event of when pigs stop shedding *Salmonella* (last positive culture)
**Figure 3.4:** Predictive probability of *Salmonella* shedding in pigs constructed against serum adjusted optical density (OD) obtained by ELISA (adjusted OD represents the amount of antibody against *Salmonella* in the serum samples)
CHAPTER 4: CONCLUSIONS

4.1 Research Summary and Conclusions

The first component of this thesis evaluated the effectiveness of in-feed flavophospholipol in reducing Salmonella shedding and antimicrobial resistance associated with Salmonella and generic E. coli. Salmonella spp. presents a public health and food safety concern due to the risk of Salmonella moving from farm to retail pork via contamination at slaughter. Flavophospholipol, an inexpensive antibiotic, is potentially a cost efficient method for farmers to mitigate Salmonella in swine. Although flavophospholipol is an antibiotic, it has no therapeutic applications in humans or veterinary medicine due to its poor pharmacokinetic and pharmacodynamic features (Butaye et al., 2003; Pfaller, 2006). It is not absorbed from the intestinal tract when given orally and it’s antimicrobial activity is limited to Gram-positive bacteria (Butaye et al., 2003; Pfaller, 2006). Furthermore, resistance to flavophospholipol in the bacterial population is slow to develop through mutations in the bacterial chromosome and is non-transferable (Kissel, 1998; Pfaller, 2006). Therefore the concerns about using antibiotics in food-producing animals do not apply to flavophospholipol.

In earlier challenge studies, flavophospholipol was found to significantly reduce Salmonella shedding and antimicrobial resistance in Salmonella Typhimurium pigs (Dealy and Moeller, 1976; Dorr and Gebreyes, 2009). However, the work contained in this thesis indicated that pigs naturally-infected with Salmonella continue to shed for several weeks whether or not they received flavophospholipol in their feed. This may in part be explained by the fact that pigs used for this work were infected with multiple serotypes, Salmonella Typhimurium and
Salmonella Livingstone being the most prevalent, causing reinfections and renewed shedding. Due to this, although the present study may have been representative of the farm situation, it is difficult to estimate the effectiveness of flavophospholipol on Salmonella shedding and antimicrobial resistance of Salmonella. However, the antimicrobial resistance pattern of commensal E. coli isolated from the pigs receiving flavophospholipol for 10 weeks were different from the antimicrobial resistance pattern of commensal E. coli from control pigs, indicating a positive influence, similar to what has been reported in the literature.

Furthermore, the Salmonella pathogen may have already established a permanent infection in the pigs before they received treatment with flavophospholipol during the nursery stage. Research suggests that flavophospholipol, by altering the microflora in favour of beneficial bacteria, inhibits the colonization of Salmonella in animals (Bolder et al., 1999). If a high degree of Salmonella colonization occurred in the gut flora prior to the pigs receiving treatment, this may have prevented flavophospholipol from having a beneficial impact. If these same pigs were provided a diet containing flavophospholipol at an earlier stage (i.e. immediately after weaning) when the pigs were younger and most susceptible to Salmonella infection, a different outcome may have occurred. Future studies are needed to investigate the preventive effect of flavophospholipol if administered during the nursery stage before pigs become infected with Salmonella and antimicrobial resistance commensal bacteria such as generic E. coli.

Understanding the dynamics and epidemiology of Salmonella infection in the swine population continues to be an important knowledge gap in our ability to improve prevention and control of Salmonella. Using the Salmonella shedding trends from Chapter 2, the second component of the thesis explored Salmonella shedding and antibody response to Salmonella
from the grower-finisher stage until slaughter. Findings revealed a high prevalence of Salmonella during the early grower-finisher stage with Salmonella shedding significantly decreasing as pigs aged. Although the estimated median of Salmonella shedding duration was 3 or 4 weeks based on our trial assumptions, pigs were found to shed Salmonella for up to 8 weeks in the grower-finisher stage. Furthermore, there was a 4-week lag period between the peak of Salmonella shedding (early grower stage) to the peak of antibody response (mid-finishing stage). Findings from the present study and previously published studies reveal variability in Salmonella shedding, duration and antibody response to Salmonella and this may be due to the differences in sampling methods, virulence of different serotypes, genetics of pigs and husbandry practices (Nielsen et al., 1995; Rajić et al., 2005). Although the present study, had an increased the probability of Salmonella detection (both shedding and antibody response), the study was limited to a small population of pigs from a single source and the findings may not being extrapolated to a broad population or compared to prevalence studies. Furthermore, the present study cannot be compared to many challenge studies that assess the prevalence of Salmonella shedding and antibody response to Salmonella in pigs colonized by one serotype.

During slaughter, Salmonella was isolated from the spleen, liver, lymph node (neck and ileocecal), tonsil, and cecal contents. The absence of Salmonella detection in fecal samples (bacteriology culturing) in pigs in the late finisher stage was not an indication that Salmonella could not be found in tissue at the time of slaughter. However, serology was able to capture intermittent shedders in the late finisher stage that were Salmonella positive at slaughter. Intermittent shedding and Salmonella colonization in tissue may be more closely associated with serotypes. Salmonella Typhimurium, which was commonly recovered at slaughter, has been
previously reported to colonize in lymph nodes and tissue (Wood et al., 1989). Whereas, other commonly recovered serotypes from fecal samples (i.e. Salmonella Livingstone) during the grower-finisher period was not found at the time of slaughter. Further research investigating the duration and extent of colonization caused by Salmonella serotypes will aid in understanding the dynamics of Salmonella infection.

Overall, this thesis reports on a small population of pigs that were highly infected with Salmonella. These pigs, from a single source population, were infected with multiple serotypes but appeared clinically healthy. Pigs were found to shed a high prevalence of Salmonella during the early grower phase with shedding declining as pigs aged. From the peak of Salmonella infection in pigs, antibody response was delayed 28 days with the peak level of antibodies occurring in mid-finishing stage. During the grower-finisher stage, flavophospholipol was used, as a control method, to reduce Salmonella and antimicrobial resistance, however was not successful under the circumstances. This could have been due to the high degree of Salmonella colonization in the gut flora prior to the pigs receiving treatment that enabled flavophospholipol from having an impact on the beneficial bacteria. One of the reasons flavophospholipol is attractive as a control for Salmonella on pig farms is that pork producers might gain some economic benefit from the use of the antibiotic if it improves growth rate and feed efficiency at the same time that it controls Salmonella shedding. Future research assessing the preventive measures of flavophospholipol on Salmonella and antimicrobial resistance in nursery pigs would be beneficial. In addition, this thesis also captured intermittent shedders and recovered Salmonella at slaughter addressing the existing food safety and public health concern. Furthering
our understanding of the epidemiology and dynamics of virulent strains of *Salmonella* is important.

### 4.2 References


Kissel, A. (1998a). Determination of the in-vitro susceptibility of selected bacterial strains carrying resistance plasmids to the feed additives salinomycin–sodium (SAL) and flavophospholipol (FPL) and to various active substances used for therapeutic purposes—MIC determina. *Germany: Hoechst Roussel Vet*.


Appendix I

Model 1: Statistical model assessing *Salmonella* shedding in treatment and control pigs from Week 1 to Week 11

```
melogit SalPig i.tx ||pen1: ||week:, nolog or
Mixed-effects logistic regression Number of obs  =  473

---
Group Variable | No. of Groups | Observations per Group | Minimum | Average | Maximum
---
pen1 | 9 | 44 | 52.6 | 55
week | 99 | 4 | 4.8 | 5
---
Integration method: mvaghermite Integration pts.  =  7

Log likelihood = -208.39958 Wald chi2(1)  = 0.05
Prob > chi2    = 0.8207

SalPig | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
---
1.tx   |   0.8598549   0.5728551  -0.23   0.821     0.2329888     3.17333
_cons  |   0.1036024   0.0587738  -4.00   0.000     0.0340786    0.3149618
---
pen1   | var(_cons)   7.84e-32  1.75e-16  .    .
pen1>week| var(_cons)  10.55922   3.753155   5.261161  21.19248
---
LR test vs. logistic model: chibar2(01) = 133.51 Prob >= chibar2 = 0.0000
```

*Multi-level mixed effect logistic regression model with pen and week modeled as a random effect*
Model 2: Statistical model assessing survival probability of *Salmonella* shedding in treatment and control pigs from Week 1 to Week 11

```
sts test (tx)
    failure _d: shed
    analysis time _t: week

Log-rank test for equality of survivor functions

<table>
<thead>
<tr>
<th>tx</th>
<th>Events observed</th>
<th>Events expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19</td>
<td>19.56</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>23.44</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>43.00</td>
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</table>

\[ \text{chi2}(1) = 0.05 \]
\[ \text{Pr}>\text{chi2} = 0.8216 \]
```

*Log rank test*
Model 3: Statistical model assessing *Salmonella* status in treatment and control pens from Week 1 to Week 11

```
melogit SalPen i.tx i.room ||pen1:, nolog or
```

Mixed-effects logistic regression

|            | Odds Ratio | Std. Err. | z   | P>|z|   | [95% Conf. Interval] |
|------------|------------|-----------|-----|------|----------------------|
| 1.tx       | 1.558159   | .9474375  | 0.73| 0.466| .4731937             | 5.130791               |
| 2.room     | .0456352   | .0250874  | -5.62| 0.000| .0155368             | .134041               |
| _cons      | .5067769   | .23161    | -1.49| 0.137| .2069169             | 1.241188              |

```
pen1          
var(_cons)    | .7011551   | .3958477  |
```

LR test vs. logistic model: chibar2(01) = 38.28 Prob >= chibar2 = 0.0000

*Multi-level mixed effect logistic regression model with pen as a random effect and room as a fixed effect*
Model 4: Statistical model assessing weight in treatment and control pigs from Week 1 to Week 11

mixed Weight i.tx ||pen1: ||week: if pig==22
Performing EM optimization:
Performing gradient-based optimization:
Iteration 0:   log likelihood = -617.07096
Iteration 1:   log likelihood = -616.97477
Iteration 2:   log likelihood = -616.97408
Iteration 3:   log likelihood = -616.97408
Computing standard errors:
Mixed-effects ML regression                     Number of obs     =        168
-------------------------------------------------------------
|     No. of
Observations per Group
Group Variable |     Groups    Minimum    Average    Maximum
----------------
|--------------------------------------------
pen1 |          9         16       18.7         20
week |         36          4          4.7          5
-------------------------------------------------------------
Wald chi2(1)      =       0.01
Log likelihood = -616.97408                     Prob > chi2       =     0.9313
------------------------------------------------------------------
Weight |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------
1.tx |   .7491903   8.689079     0.09   0.931     -16.28109    17.77947
_cons |   51.33592   6.475902     7.93   0.000     38.64338    64.02845
------------------------------------------------------------------
Random-effects Parameters |   Estimate   Std. Err.     [95% Conf. Interval]
----------------------------
pen1: Identity                |   var(_cons) |  3.24e-15   5.63e-14      5.08e-30    2.064228
week: Identity                |   var(_cons) |  663.6557   158.2119     415.9308    1058.923
                               |   var(Residual) |  34.55045   4.252951     27.14413    43.97759
LR test vs. linear model: chi2(2) = 342.87                     Prob > chi2 = 0.0000
Note: LR test is conservative and provided only for reference.

*Mixed linear regression model with pen and week as a random effect and pig 22 removed
Model 5: Statistical model assessing average daily gains in treatment and control pigs from Week 1 to Week 11

mixed Adg i.tx ||pen1: ||week: if pig==22

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0:   log likelihood =  33.376286
Iteration 1:   log likelihood =   33.57349
Iteration 2:   log likelihood =  33.579018
Iteration 3:   log likelihood =  33.579021

Computing standard errors:

Mixed-effects ML regression

Number of obs = 126

Observations per Group

<table>
<thead>
<tr>
<th>Group Variable</th>
<th>No. of Groups</th>
<th>Minimum</th>
<th>Average</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pen1</td>
<td>9</td>
<td>12</td>
<td>14.0</td>
<td>15</td>
</tr>
<tr>
<td>week</td>
<td>27</td>
<td>4</td>
<td>4.7</td>
<td>5</td>
</tr>
</tbody>
</table>

Wald chi2(1) = 0.00
Prob > chi2 = 0.9731

Log likelihood = 33.579021

| Adg  | Coef.   | Std. Err. | z    | P>|z| | [95% Conf. Interval] |
|------|---------|-----------|-----|-----|---------------------|
| 1.tx | 0.0028305 | 0.0838215 | 0.03 | 0.973 | -0.1614566, 0.1671176 |
| _cons| 0.9559823 | 0.0624310 | 15.31| 0.000 | 0.8336198, 1.078345  |

<table>
<thead>
<tr>
<th>Random-effects Parameters</th>
<th>Estimate</th>
<th>Std. Err.</th>
<th>[95% Conf. Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pen1: Identity</td>
<td>var(_cons)</td>
<td>8.07e-16</td>
<td>.</td>
</tr>
<tr>
<td>week: Identity</td>
<td>var(_cons)</td>
<td>0.0423618</td>
<td>0.0127687, 0.0234642</td>
</tr>
<tr>
<td></td>
<td>var(Residual)</td>
<td>0.0207719, 0.0029524</td>
<td>0.0157215, 0.0274449</td>
</tr>
</tbody>
</table>

LR test vs. linear model: chi2(2) = 76.62
Prob > chi2 = 0.0000

Note: LR test is conservative and provided only for reference.

*Mixed linear regression model with pen and week as a random effect and pig 22 removed
Model 6: Statistical model assessing antimicrobial resistance in ampicillin between treatment and control pigs at Week 1 and Week 11 in *E. coli* isolates

\[
\text{xi: melogit AMP i.TX i.EVENT i.room ||pen: , nolog or}
\]
\[
i.TX \quad \_ITX_0 - 1 \quad \text{(naturally coded; _ITX_0 omitted)}
\]
\[
i.EVENT \quad \_IEVENT_1 - 2 \quad \text{(naturally coded; _IEVENT_1 omitted)}
\]
\[
i.room \quad \_Iroom_1 - 2 \quad \text{(naturally coded; _Iroom_1 omitted)}
\]

Mixed-effects logistic regression

<table>
<thead>
<tr>
<th>Group variable: pen</th>
<th>Number of obs = 65</th>
<th>Number of groups = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obs per group:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>avg</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>max</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Integration method: mvaghermite

<table>
<thead>
<tr>
<th>Integration pts. = 7</th>
</tr>
</thead>
</table>

Log likelihood = -31.769468

<table>
<thead>
<tr>
<th>Wald chi2(3) = 12.82</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prob &gt; chi2 = 0.0050</td>
</tr>
</tbody>
</table>

| AMP  | Odds Ratio | Std. Err. | z    | P>|z| | [95% Conf. Interval] |
|------|------------|-----------|------|------|---------------------|
| _ITX_1 | 0.2245558 | 0.1496446 | -2.24| 0.025| 0.0608249 - 0.8290243 |
| _IEVENT_2 | 6.139156 | 4.675048 | 2.38 | 0.017| 1.38009 - 27.30926 |
| _Iroom_2 | 2.99493 | 3.696524 | 0.89 | 0.374| 0.2665521 - 33.65047 |
| _cons | 2.228362 | 1.201382 | 1.49 | 0.137| 0.7745958 - 6.410563 |

| pen | var(_cons) | 2.22e-33 | 3.26e-17 | . | . |

| LR test vs. logistic model: chi2(0) = 0.00 | Prob > chi2 = . |

Note: LR test is conservative and provided only for reference.

*Logistic regression model with event as a fixed effect*
Model 7: Statistical model assessing antimicrobial resistance in chloramphenicol between treatment and control pigs at Week 1 and Week 11 in *E. coli* isolates

```
xi: melogit CHL i.TXT i.EVENT i.room ||pen: , nolog or
  i.TXT         _ITX_0-1 (naturally coded; _ITX_0 omitted)
i.EVENT     _IEVENT_1-2         (naturally coded; _IEVENT_1 omitted)
i.room       _Iroom_1-2        (naturally coded; _Iroom_1 omitted)
Mixed-effects logistic regression
Group variable: pen
Number of obs     =         65
Number of groups  =          9
Obs per group:
  min =          2
  avg =        7.2
  max =         10
Integration method: mvaghermite
Integration pts. =          7
Log likelihood =   -36.37846
Wald chi2(3)    =       6.82
Prob > chi2       =     0.0780

------------------------------------------------------------------------------
CHL | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
------------------------------------------------------------------------------
      _ITX_1 |   .2458017   .1409249     -2.45   0.014     .0799039    .7561398
      _IEVENT_2 |   1.483286   .9635676     0.61   0.544     .4152123    5.298827
      _Iroom_2 |   .4481513   .3814591    -0.94   0.346     .0845076    2.376586
      _cons   |   .8895932   .4212266     0.25   0.805     .3516767    2.250295
------------------------------------------------------------------------------
pen
  var(_cons) |   5.30e-35   4.35e-18
------------------------------------------------------------------------------
LR test vs. logistic model: chi2(0) = 0.00    Prob > chi2 = .
Note: LR test is conservative and provided only for reference.
```

*Logistic regression model with event as a fixed effect*
Model 8: Statistical model assessing antimicrobial resistance in trimethoprim/sulfamethoxazole between treatment and control pigs at Week 1 and Week 11 in *E. coli* isolate

```plaintext
xi: melogit SXT i.TX i.EVENT i.room ||pen: , nolog or
  i.TX          _ITX_0-1 (naturally coded; _ITX_0 omitted)
i.EVENT        _IEVENT_1-2 (naturally coded; _IEVENT_1 omitted)
i.room         _Iroom_1-2  (naturally coded; _Iroom_1 omitted)

Mixed-effects logistic regression

Number of obs = 65
Group variable: pen Number of groups = 9

Obs per group:
  min = 2
  avg = 7.2
  max = 10

Integration method: mvaghermite Integration pts. = 7

Wald chi2(3) = 9.41
Log likelihood = -23.137245
Prob > chi2 = 0.0243

|          | Odds Ratio | Std. Err. | z    | P>|z|  | [95% Conf. Interval] |
|----------|------------|-----------|------|-----|---------------------|
| _ITX_1   |   0.0957105|   0.0827242| -2.71|0.007| 0.0175894 0.5207961 |
| _IEVENT_2|   4.597033 |   4.022639 | 1.74 |0.081| 0.8272424 25.54597  |
| _Iroom_2 |   0.8238379|   0.7764129| -0.21|0.837| 0.1299094 5.22448   |
| _cons    |   0.2275692|   0.1469423| -2.29|0.022| 0.0641933 0.8067472 |

pen
  var(_cons) | 4.05e-32 2.53e-16 . .

LR test vs. logistic model: chi2(0) = 0.00  Prob > chi2 = .

Note: LR test is conservative and provided only for reference.

*Logistic regression model with event as a fixed effect*
Appendix II

Model 1: Statistical model assessing *Salmonella* shedding as pigs age from 10 weeks of age to 20 weeks of age

```
xi: melogit SalPig age_cat1 age_cat2 ||pen1: ||week:, nolog

Mixed-effects logistic regression

Number of obs = 479

---------------------------------
<p>|     No. of | Observations per Group |</p>
<table>
<thead>
<tr>
<th>Group Variable</th>
<th>Groups    Minimum    Average    Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pen1</td>
<td>9         47       53.2         55</td>
</tr>
<tr>
<td>week</td>
<td>99          4        4.8          5</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------</td>
</tr>
</tbody>
</table>

Integration method: mvaghermite
Integration pts. = 7

Log likelihood = -174.60539
Wald chi2(2) = 57.04
Prob > chi2 = 0.0000

------------------------------------------------------------------------------
<p>|        SalPig | Odds Ratio | Std. Err. | z     | P&gt;|z| |  [95% Conf. Interval] |
|---------------|------------|-----------|-------|------|-----------------------|
| age_cat1      | 18.4817    | 17.63244  | 3.06  | 0.002| 2.848749   119.9028 |
| age_cat2      | .0018902   | .0018891  | -6.27 | 0.000| .0002666    .0134027 |</p>
<table>
<thead>
<tr>
<th>_cons</th>
<th>1.557917</th>
<th>.6845619</th>
<th>1.01</th>
<th>0.313</th>
<th>.6584442    3.68612</th>
</tr>
</thead>
<tbody>
<tr>
<td>pen1 var(_cons)</td>
<td>.2833836</td>
<td>.3275692</td>
<td>.0294071</td>
<td>2.730845</td>
<td></td>
</tr>
<tr>
<td>pen1&gt;week var(_cons)</td>
<td>1.738007</td>
<td>.7826157</td>
<td>.7190512</td>
<td>4.200909</td>
<td></td>
</tr>
</tbody>
</table>

LR test vs. logistic model: chi2(2) = 21.02
Prob > chi2 = 0.0000

Note: LR test is conservative and provided only for reference.  

*Multi-level mixed effect logistic regression model with age as ordinal data (10-11 weeks of age referent, 11 to 12 weeks of age as age_cat1, 12 to 20 weeks of age as age_cat2) with pen and week modeled as a random effect*
Model 2: Statistical model assessing seropositivity in relation to *Salmonella* shedding from Week 1 to Week 11

<table>
<thead>
<tr>
<th>Group Variable</th>
<th>No. of Groups</th>
<th>Minimum</th>
<th>Average</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pen1</td>
<td>9</td>
<td>16</td>
<td>18.0</td>
<td>20</td>
</tr>
<tr>
<td>week</td>
<td>36</td>
<td>2</td>
<td>4.5</td>
<td>5</td>
</tr>
</tbody>
</table>

Integration method: mvaghermite
Integration pts. = 7

Wald chi2(1) = 4.04, Prob > chi2 = 0.0444

|               | Odds Ratio | Std. Err. | z      | P>|z|   | [95% Conf. Interval] |
|---------------|------------|-----------|--------|-------|---------------------|
| _ISalPig_1    | 0.3781926  | 0.1828973 | -2.01  | 0.044 | 0.1465765 - 0.975802 |
| _cons         | 3.080113   | 0.9583097 | 3.62   | 0.000 | 1.673917 5.667602   |
| pen1 var(_cons) | 0.0585809  | 0.3420623 | 6.27e-07 | 5470.724 |
| pen1>week var(_cons) | 1.019283 | 0.9006618 | 0.1803658 | 5.760177 |

LR test vs. logistic model: chi2(2) = 4.73, Prob > chi2 = 0.0940

Note: LR test is conservative and provided only for reference.

*Multi-level mixed effect logistic regression model with pen and week modeled as a random effect