ANTIMICROBIAL SUSCEPTIBILITY PROFILES IN *E. COLI* POPULATIONS FROM GROWING PIGS FOLLOWING USE OF FEED-BASED SUBTHERAPEUTIC ANTIMICROBIALS OR A COMPETITIVE EXCLUSION CULTURE

by

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(Under the Direction of Barry G. Harmon, DVM, PhD)

ABSTRACT

In view of the significant concerns regarding increasing antimicrobial resistance and the need for efficacious alternatives to the use of antimicrobials, the studies presented were based on two primary objectives: 1) To determine the effect of three feed-based subtherapeutic antimicrobials (FSAs; apramycin, chlortetracycline, and carbadox) and 2) the effect of porcine-derived mucosal competitive exclusion (PCE) culture on both the antimicrobial susceptibility profiles of commensal *E. coli* populations and animal performance in growing pigs.

For Objective 1, three replicate trials were conducted using growing piglets fed standard diets with and without subtherapeutic antimicrobials. For Objective 2, two replicate trials were coducted with a porcine-derived competitive exclusion culture replacing the subtherapeutic antimicrobials. Fecal samples were cultured for commensal *E. coli* at regular intervals from all piglets and antimicrobial susceptibility profiles of *E. coli* isolates were determined. Animal performance was also measured.

Results from Objective 1 indicated significantly increased antimicrobial resistance in *E. coli* isolates from piglets fed FSAs when compared to controls ($P < 0.0001$). However,

resistance levels returned to baseline by the end of each trial. Piglets fed FSAs demonstrated higher average daily gains at Days 61 and 75 (Day 61: weight at end of carbadox-starter diet, Day 75: weight at end chlortetracycline grower diet) ($P < 0.001$) and improved feed efficiency at Day 75 ($P < 0.0001$).

Results from Objective 2 indicated that streptomycin resistance in *E. coli* from piglets treated with PCE culture was variable, initially increasing in PCE-treated piglets subsequent to the first 2 doses (Days 0-1, $P < 0.0001$) and then returning to baseline levels by Day 21 (weaning). Significant differences for streptomycin were observed again after the second 2 doses at weaning (Days 21-22, $P <0.0001$). Resistance in *E. coli* to tetracycline remained consistent over time for both groups. While a significant increase in tetracycline resistance for PCE-treated piglets when compared to controls was noted ($P < 0.0001$), the contribution of tetracycline resistance from sows and the environment was unclear. Piglets treated with the PCE culture demonstrated improved feed efficiencies when compared to control piglets ($P < 0.005$).

These data suggest that some antimicrobials or classes of antimicrobials may not lead to the development of persistently resistant *E. coli* populations and may therefore be suitable for continued use. Additionally, data suggest that PCE cultures improve animal performance and increased resistance in *E. coli* appeared to be transient for some antimicrobials. Therefore, antimicrobial susceptibility testing of PCE cultures should be performed. Further research is warranted on the populations of resistant *E. coli.*

INDEX WORDS: antimicrobial resistance, *E. coli*, animal feed, subtherapeutic antimicrobials, swine, pig, competitive exclusion, performance

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DEDICATION

This dissertation is dedicated to Squirt, who has been my steadfast companion for 18 years; to John, whose unwavering encouragement, support and assistance allowed me to complete this adventure; and finally to my parents, without whom I could never have come so far. Peace, love and light to all.

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CHAPTER 1

INTRODUCTION

Antimicrobial resistance is a global concern in food safety today. Increased antimicrobial resistance has been observed among zoonotic pathogens and commensal flora from humans and animals. Since the discovery of penicillin in the 1940's, antimicrobial usage in humans and livestock has increased dramatically worldwide. Use in livestock increased with the advent of confinement agriculture[1] and research in 1946 demonstrated increased animal productivity following use of subtherapeutic levels of antimicrobials in animal feeds.[2] Increased antimicrobial resistance in response to increased antimicrobial use in animal production was recognized early on and guidelines have been proposed since the 1960's in attempts to promote appropriate use in humans and livestock.[3]

Feed-based subtherapeutic antimicrobials (FSAs) have been in use in animal production for nearly 50 years and their efficacy is well documented.[4-8] Continued concerns regarding an apparent association between the use of FSAs in food animal production and a concomitant increase in antimicrobial drug resistance among zoonotic enteropathogens have provided the impetus to ban the use of FSAs in animal feeds.[9-12] In Europe, bans against FSAs such as avoparcin and virginiamycin invoking the precautionary principle are already in place.[13-15] The potential benefits versus risks of FSA use are currently being debated in the United States.[4,16,17] Recent reports suggest that antimicrobial resistant foodborne infections may be associated with higher morbidity compared to infection with susceptible pathogens.[18,19] However, the correlation between use of FSAs and the impact on human health remains unclear.[1,20,21]

Escherichia coli (*E. coli*) strains may be commensal or pathogenic. They are found in the digestive tract of most animals and are ubiquitous in the environment. The occurrence and persistence of antimicrobial resistance in both commensal and pathogenic *E. coli* has been documented,[22-26] and a recent report suggests that any *E. coli* strain acquiring the appropriate virulence factors may become pathogenic regardless its of antimicrobial susceptibility profile.[27] Part of the commensal flora, *E. coli* are thought to be reservoirs of resistance genes capable of transferring genes to foodborne and other zoonotic pathogens.[9]

The swine industry is a major consumer of FSAs,[7,28] and studies have shown increases in antimicrobial resistance in enteric bacteria isolated from operations using FSAs.[29,30,31] Epidemiologic studies suggest that enteric flora from pigs often demonstrate resistance to antimicrobials such as tetracycline, streptomycin, and sulfonamides which have been used in both human and animal medicine for over 40 years.[32-34]

While use of antimicrobial drugs in livestock production has made a significant impact on animal health, welfare, and productivity, interest in suitable alternatives such as pre/probiotics, organic acids, and use of cultures of normal flora or "competitive exclusion" (CE) cultures from young animals has increased significantly in the wake of the antimicrobial resistance issue.[35] Studies evaluating the efficacy of these alternatives are ongoing; however, to date, no single intervention has demonstrated results comparable to the use of subtherapeutic antimicrobials. Nurmi et al. demonstrated efficacy of inoculating chicks with unmanipulated intestinal bacteria from adult birds in preventing infection when challenged with *Salmonella.*[36] Colonization resistance to bacterial pathogens in the intestine is thought to be mediated by the anaerobic population of intestinal bacteria.[37] Competitive exclusion cultures are comprised in large part of anaerobic and facultative populations of bacteria, and their efficacy has been

demonstrated in this respect.[38-40] Performance of animals receiving probiotics has been variable.[35,41] Data regarding the effect of CE on animal productivity is not readily available. Additionally, there is little information regarding the effect on antimicrobial resistance in bacterial populations following use of probiotics or CE cultures.

Current literature suggests that a successful move from subtherapeutic antimicrobial use to suitable alternatives will require a combination of precise nutrition, proper management and sanitation, careful record keeping, and appropriately applied supplements at key points in growth.[41,42] A recent epidemiologic report proposes that subtherapeutic antimicrobials be limited to use during the nursery phase in swine production where an optimal effect has been demonstrated. Limiting subtherapeutic antimicrobial use to this phase in swine production would have a major impact on the amount of antimicrobials used. Use of some subtherapeutic antimicrobials combined with strategic supplementation of alternatives such as probiotics and CE cultures may allow for additional decreases in antimicrobial use in swine production without sacrificing animal health, welfare and productivity.

In view of the limited information available on controlled use of FSAs and the effect of alternatives to FSAs the studies presented herein evaluated the antimicrobial susceptibility of commensal *E. coli* populations and animal performance in growing piglets either fed three feed-based subtherapeutic antimicrobials (FSAs; apramycin, carbadox, and tetracycline), or orally treated with a porcine-derived competitive exclusion (PCE) culture.

CHAPTER 2

LITERATURE REVIEW

1. Commensal flora of swine

The development of the porcine intestinal flora in neonates is influenced by the immediate environment (sow, teats, pen)[43,44] and then appears to develop independently of the external environment within days to the first week post-farrowing.[43,45] While stress and illness can alter the succession of intestinal flora,[45,46] minimal changes have been reported in the intestinal flora of weaned piglets maintained under the same conditions.[47] In general, progression of bacterial populations has been reported as initial colonization by coliforms, streptococci and *Clostridium*, followed by slower colonization by lactobacilli.[48,49] However, recent reports describing attempts to characterize anaerobic populations suggest that there is a large population yet to be defined.[50,51] In the healthy piglet intestine, the coliform *Escherichia coli*, decreases within the first few weeks as the colonization of predominantly anaerobic bactera increases. At weaning, the relatively stable enteric population may be temporarily disrupted and/or altered with the conversion to solid food resulting in changes in enterocyte morphology, fluid balance and electrolytes, pH, enzymatic activity, and motility.[48,49]

The diverse gram-negative bacterium, *E. coli* has been studied extensively. Although present as part of the commensal flora in pigs[52,53] there are over 2,000 serotypes of *E. coli* including many that are pathogenic to humans and animals. Studies investigating biochemical profiles,[54] genomics,[55] and proteomics[56] have been performed in *E. coli.* These bacteria have been used in vaccine development,[57,58] for production of proteins,[59] as hosts for genetic exchange,[60-62] and as a novel probiotic to treat disease in cattle caused by another serotype (*E.*

coli 0157:H7)[63,64] demonstrating the diverse applications of this species. *Escherichia coli* have also been widely used as a sentinel organism for monitoring the development of antimicrobial resistance in humans and animals.[65-70]

To date, there remains a paucity of information regarding the comprehensive characterization of bacterial species present in the mammalian intestine. The largely anaerobic milieu in the intestine has yet to be defined, and one author states, "[it is] dominated by thick layers of complex bacterial flora…"[71] Research has determined that the commensal flora plays a vital role in maintaining the health and nutritional status of an animal. [72] The complex relationship between the resident flora and the mucosal immune system provides a healthy animal the ability to suppress or prevent enteric colonization of pathogenic bacteria.[37,73] Commensal flora present in the intestinal lumen produce a wide variety of nutrients such as short-chain fatty acids, amino acids, growth factors and vitamins that are used by the host.[74]

Changes made in the diet can influence the population of commensal flora and thus the fermentative activities.[48,49] Studies investigating diet-modification to alter resident flora and improve nutrient utilization include the use of organic acids and herbs.[35,41,42] Organic acids used to acidify the diet have also been in use for several years to decrease the stomach pH of weaned piglets which favors growth of beneficial bacteria such as lactobacilli.[75-77] With the increasing concern of antimicrobial resistance, probiotics (beneficial enteric bacteria such as lactic acid bacteria, bifidobacteria, and enterococci) and competitive exclusion cultures (populations of enteric bacteria derived from the animal species of interest) are under intensive investigation for their ability to decrease disease, promote growth, and reduce the need for antimicrobials in animal production.[35,41,78] Probiotics have been used in animal production with varying success for nearly 20 years.[35,41] New research may improve upon current products by identifying the

most beneficial organism(s), combining probiotic organisms with prebiotics (feed ingredients such as oligosaccharides that provide nutrition for desirable organisms) and/or determining appropriate doses and treatment duration(s) to encourage optimal colonization of appropriate commensal flora.

2. Mucosal immunity in the gut

Physical barriers exist within the host to protect it from its environment. These barriers include the skin and the mucosae of the intestinal and respiratory tracts. The extensive surface area of the mucosae includes a complex and effective immunological defense while allowing for selective absorption of essential substances, provides tolerance to commensal microflora, food and environmental antigens, and acts as a barrier to colonization of potential pathogens. Though all protective aspects are not fully understood, the major components of enteric mucosal immunity include gut-associated lymphoid tissue (GALT), secretory immunoglobulin A (IgA), and a plethora of enzymes, cytokines and other endogenous antimicrobial peptides.[79]

One of the largest lymphoid organs in the body, the GALT structures are distributed throughout the intestine and include the aggregate nodules known as Peyer's patches.[80,81] Typically located along the antimesenteric side of the small intestine, Peyer's patch follicles decrease with age.[80] In contrast to other lymph nodes of the body, these follicles have less organized structure and no afferent lymphatic ducts. Peyer's patches represent the primary inductive sites (antigen exposure) for T and B cells. The mature cells then migrate to the intestinal lamina propria, the main effector site. Many studies have been performed in swine to more fully understand the emigration of lymphocytes from GALT as pigs are often used as a model for human physiology.[81-83] Results from these studies suggest that the complex microenvironment of the intestine has diverse influences such as the production of bone-marrow

derived precursor cells and immunoglobulin and T-cell receptor gene rearrangement in both B and T-cell lineages.

M-cells are found in the follicle-associated epithelium around Peyer's patch follicles and are an essential component in antigen sampling.[80,81] Enfolded in the basolateral membrane of these cells are lymphocytes, macrophages, plasma cells and the occasional polymorphonuclear leukocyte, responding to the antigenic sampling activity of the M-cells. In addition, the surface of M-cells contains secretory IgA binding sites. While these cells allow for efficient sampling of noninvasive pathogens such as *Vibrio cholerae*, the transepithelial pathway they provide is used by many invasive pathogens such as *Salmonella* Typhimurium. In the case of *S.* Typhimurium, a strong mucosal immune response is mounted; however, it arrives too late to prevent systemic spread to other organs. From M-cells, antigen-presenting cells (APCs) in the GALT continue the induction of the response.[81,84,85] In pigs, Haverson, et al.[85] demonstrated that differential antigen presentation is accomplished by a heterogeneous population of APCs, including dendritic cells, macrophages, CD4+ T cells and B cells.

The intestinal lamina propria is a primary effector site where IgA+ cells predominate.[83,86] Antigen-stimulated B cells are induced by T-helper cells to switch to IgA class antibody production and then migrate to the lamina propria where differentiation takes place. Both IgM and IgA are transported across the epithelium via membrane-bound vesicles and provide first line defenses against mucosal pathogens. Although peripheral blood B cell switch differentiation to various IgG isotypes requires specific factors or cytokines, a specific secretory IgA switch factor has not been identified.[83] Sonoda et al.[87] used transforming growth factor β to induce a switch from IgM to IgA production. More recently, Butler et al.[88] suggested *de novo* synthesis of IgA in the fetal porcine thymus.

As aforementioned, lymphocyte trafficking has been extensively studied in swine. In addition to migrating from blood to lymphoid tissues, swine lymphocytes migrate to most other tissues as well. Mammary glands of sows contain lymphocytes from both GALT and peripheral lymph nodes.[89] Immunoglobulins are secreted into the colostrum. Through this passive transfer the piglet obtains it's only source of immunoglobulins as there is virtually no transfer of antibody prior to birth.[86] Colostral lymphocytes are also absorbed; however, it is not clear if these lymphocytes confer significant cell-mediated immunity to the piglets. Although piglets have a fully functional immune system at birth, they are immunologically naïve and at risk for infection because they have not previously been exposed to any antigens. Once antigenic exposure occurs, it takes 7-10 days to develop a primary antibody or cell-mediated response. During the first days of life the IgM isotype is most abundant, but there is a progressive shift to IgA as the animal matures.[90] Additionally, the antibody repertoire development in the neonatal piglet is either highly restricted or almost entirely determined by diversity in a single region.[91]

Research using germ-free animal models has revealed significant differences in the development of mucosal immunity between conventionally raised and germ-free animals.[92] Immunologic effects of commensal microflora are complex and include: 1) the induction of major histocompatibility complex class II (MHC II) molecules in the small intestinal epithelial cells; 2) expansion and acquisition of cytotoxic activity in intraepithelial lymphocytes in the small intestine; 3) full development of Peyer's patches; 4) expansion of IgA-producing cells in the lamina propria; 5) enhanced production of superoxide anion in neutrophils and macrophages; and 6) induction and maintenance of oral tolerance in antibody response.[93] Previous studies using commensal bacteria singly or even from *in vitro* mixtures[94] to stimulate IgA in formerly germ-free animals failed to produce the same IgA levels observed in conventionally raised

animals. However, inoculation of formerly germ-free mice with segmented filamentous bacteria has resulted in production of significantly higher IgA levels.[92,95]

When a novel antigen is encountered in the gut, one of three main responses can be expected: 1) local secretion of IgA; 2) systemic immune response generating serum antibodies and cell-mediated immunity; or 3) a state of immunologic unresponsiveness (tolerance) which can prevent inappropriate immune responses to antigens that are not a threat to the host upon subsequent re-exposure to the antigen.[96,97] Pathogens and toxins induce the first two responses, while environmental antigens and commensal bacteria tend to induce tolerance. Precise mechanisms leading to both the induction and maintenance of tolerance, and how the determination is made between resultant tolerance versus inflammation remain unclear. Factors that may influence tolerance include the nature of the antigen, the amount and frequency of antigen presentation, and host factors such as health, age, background genetics, nutrition, and status of commensal flora.[96]

The intimate and complex relationship between the mucosal immune system and the resident flora of the gut provides an essential barrier to pathogens as well as immune stimulation of other body systems. Recent advances in the use of probiotics, "living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition,"[98] have demonstrated benefits to patients with acute and chronic gastrointestinal illnesses,[98-101] atopy,[102-105] arthritis,[106,107] and cancer.[98,108] Studied for nearly a century, mechanisms by which probiotics enhance immunity are only recently being discovered. Recent research has established the importance of selecting the appropriate probiotic organism by identifying desirable properties such as ability to colonize the gut, resistance to acid and bile, production of antimicrobial substances, and demonstrated beneficial health effects such as

immunmodulation.[98,109-111] Probiotic use in animal production has increased in popularity concurrent with mounting concerns of antimicrobial use and resistance[16,17] and continues to be a growing area of research.

3. Bacterial enteric pathogens of swine: *Salmonella*

While advancements in modern livestock production have helped to increase overall animal health and production, zoonotic enteropathogens continue to be transmitted through the food chain. As a result, foodborne illness remains a significant cause of morbidity and mortality in the US and worldwide.[112] In swine production, bacterial pathogens of human health concern include: *Salmonella* species, *Campylobacter jejuni*,[113,114] Enterohemmorhagic *Escherichia coli* (EHEC),[115] *Yersinia enterocolitica*,[116] *Clostridium botulinum*,[117] *Listeria monocytogenes*,[118] *Staphylococcus aureus*,[119] *Helicobacter*[120] and *Arcobacter* species.[121] Many of these bacterial species exert no ill effects on their porcine hosts, but may be serious human pathogens. *Salmonella* species are a common source of disease in humans.[122]

Salmonellae are gram-negative motile bacteria found in the intestinal tract of animals. Salmonellae are able to survive for more than 12 months in the environment[123] and readily replicate in meat[124] and in feedstuff.[125] Virtually all vertebrates are believed to be susceptible to infection with *Salmonella*.[126] There are more than 2400 serovars of *Salmonella enterica* and some are adapted to single host species, such as *S.* Choleraesuis adapted in swine and *S.* Dublin in cattle. Other serovars have a broader host range. For example, *S.* Typhimurium infections occur in many mammals and reptiles.

In swine, the epidemiology of *Salmonella* includes the clinical manifestation of salmonellosis in swine, in addition to contamination of pork carcasses and retail products which have been implicated in foodborne illness in humans.[126] Clinical outbreaks of *Salmonella* often

occur in intensively-reared weaned pigs.[126] Although disease in adults and suckling pigs is infrequent, colonization is not.[127] Subclinical shedding of *Salmonella* is commonly seen in pigs and a carrier state is often observed both after acute illness and in asymptomatic swine.[127] Colonized, shedding pigs and contaminated environments are the major sources of new infection of *Salmonella* in swine herds.[128] Clinical signs of salmonellosis are associated with enterocolitis and/or septicemia. Pigs surviving acute septicemia may develop complications including pneumonia, hepatitis, enterocolitis and occasionally meningoencephalitis due to *Salmonella*.[126]

The prevalence of *Salmonella* on pig carcasses ranges from 0% to 70% as reported by several studies carried out in different countries.[129-131] In the U.S. the prevalence of *Salmonella* on pig carcasses was 0% to 4.4%.[132] Contamination often occurs at slaughter as increased shedding and cross contamination have been demonstrated with transportation and lairage.[131,133-136] Efforts to decrease carcass contamination are now commonplace at both the farm and abattoir.[124,137,138] Treatment and prevention of *Salmonella* at the farm level includes the use of both in-feed antimicrobials and parenteral antimicrobial use.[139-141] However, the apparent association between the use of feed-based subtherapeutic antimicrobials and subsequent selection for antimicrobial resistance in foodborne pathogens,[11,12,142,142-144] as well as commensal flora such as *E. coli*[23-25,145-148] demonstrates the need for alternatives. Additionally, recent data suggest that antimicrobial resistant foodborne infections may be associated with higher morbidity when compared to more susceptible pathogens in humans.[10,18]

Alternatives to antimicrobial use in swine for treatment and prevention of *Salmonella* include vaccines, organic acids, prebiotics, probiotics, and competitive exclusion cultures (CE). Vaccination has been successful in experimental trials for *S.* Typhimurium and *S.* Cholerasuis, with limited cross-protection to other strains.[149-152] Organic acids such as fumaric and citric acid

have been added to feed to reduce acid intolerant species such as *E. coli* and *Salmonella*.[41,78,153] However, bacteria may develop acid-resistance when exposed to acidic environments over time.[41] Prebiotics such as oligosaccharides are purported to reduce the ability of certain pathogenic bacteria to adhere to the intestinal wall, as well as to stimulate phagocytic activity and proliferation of B-lymphocytes.[35,154,155] Probiotics and CE cultures have shown perhaps the most consistent results for decreasing *Salmonella* in swine[38,40,156] and poultry;[157-161] however, no single intervention has yet demonstrated results comparable to the use of antimicrobials. Reducing *Salmonella* in swine herds without the use of antimicrobials will likely require a combination of nutrition, management and sanitation solutions in addition to the incorporation of alternatives.

4. Antimicrobial use in animal feed

Subtherapeutic antimicrobials have been routinely used in animal feeds during the past 50 years. The usefulness of these drugs includes prevention of disease and growth promotion.[7,8] During the past 15 years, use of therapeutic and subtherapeutic antimicrobial drugs in food animal production has come under close scrutiny. Studies have been conducted to assess the effect of antimicrobial drugs used in animal production on the development of antimicrobial resistance in enteric organisms. [24,29,68,162,163] These studies suggest that use of feed based subtherapeutic antimicrobials (FSAs) is more often associated with increased antimicrobial resistance when compared to animals receiving no antimicrobials, or those receiving only therapeutic antimicrobials. The demonstration of antimicrobial resistant gene transfer among bacterial species[1,20,21] has further increased concerns that the commensal flora of food animals may serve as a reservoir of antimicrobial resistance.[12,29,34,68,162,164-170] The swine industry is a

major consumer of FSAs,[7,28,171] and studies have shown increases in antimicrobial resistance from operations that use FSAs compared to those that do not.[21,26,29-31,147,172-175]

Tetracycline has been in use in animal production since the 1950s, and resistance among commensal fecal organisms of food animals is common.[17,24,26] Recent data show that in humans, 80% of colonic bacteria are resistant to tetracycline.[176] While this is cause for concern as it suggests a doubling in the resistant bacteria over the past 30 years,[176] consideration must be given to the widespread use of tetracycline in humans. Additionally, DNA analysis of tetracycline resistance genes suggests that it is horizontal transfer of the tetracycline gene, not mutation, which is primarily responsible for the increase.[176] Possible explanations for the increase and persistence in tetracycline resistance in humans and animals lies not only in the wide variety and number of genetic elements that can confer tetracycline resistance (various *tet* genes, *cml*A, *omp*F) but also to the continued therapeutic and subtherapeutic use of tetracyclines. Removal of the selective pressure indicates that the total percent tetracycline-resistant population can decrease as noted in a swine herd after removal of all antimicrobials.[173] However after 126 months, resistance in enteric organisms from swine on that farm only decreased from ~75% to 44%. This study suggests that the tetracycline resistance is persistent, that a portion of the persistence is unaffected by tetracycline use, and that the prevalence is unlikely decrease to zero percent.

Sulfonamides and streptomycin also have a similar history. Sulfonamides have been in use for the longest period of time, since the 1940s,[177] and the cumulative use over time likely has had an effect on the development and persistence of resistance. However, unlike tetracycline, the resistance levels to these antimicrobials in foodborne and commensal bacterial are considerably lower.[178] Streptomycin has been in use since the 1950s and like tetracycline,

resistance may be conferred by one of several enzyme pathways. Studies in swine often indicate a high degree of resistance to streptomycin.[33,34,179] Streptomycin resistance in bacteria from swine tends to be higher than that for sulfonamides, but lower than the levels for tetracycline. In contrast, apramycin which has only been in use approximately 25 years and is not used in humans has only one mechanism of resistance.[180] The study of resistance to apramycin provides information regarding resistance levels in an antimicrobial that is not used in humans. Data indicate that though resistance to apramycin occurs rapidly following use, it does not persist once the antimicrobial is removed.[181] As apramycin is not used in humans, this information suggests that human antimicrobial use of Synercid may play a role in the development and persistence of resistant bacteria.

Carbadox is a commonly used FSA in U.S. swine herds but has been banned in Europe and Canada because of potential teratogenic and carcinogenic effects[182,183] and further investigation into the genotoxicity of carbadox should be pursued.[139] Typically used in the early growth stages,[28] carbadox has been reported to be effective against *Salmonella* and *Serpulina hyodysenenteriae*, the major cause of swine dysentery.[184,185] Previous reports have suggested that resistance to carbadox is uncommon among enteric bacteria.[68,186]

In a recent study, the effect of different antimicrobial regimes was evaluated in swine.[187] Weaned piglets with no prior exposure to antimicrobials were separated into groups and challenged intranasally with an antimicrobial-sensitive strain of *S.* Typhimurium isolated from a confirmed case of swine salmonellosis. Various in-feed antimicrobial regimes served as the treatments, with one control group receiving no antimicrobials. Treatments lasted 14 days and began day 7 post-challenge. *Escherichia coli* and *Salmonella* were recovered from each piglet over time and tested for resistance to gentamicin, apramycin, neomycin, and sulfamethazine.

Rotation with similar classes of antimicrobials resulted in the greatest resistance in *E. coli* to the antimicrobial tested, and pulse-dosing the least. However, no treatments affected the sensitivity of the *S.* Typhimurium isolates. These results differed from prior work[188] in which the same challenge strain demonstrated slight increases in resistance after treatment with apramycin. Additionally, a field study evaluating the use of subtherapeutic antimicrobials on the prevalence of antimicrobial-resistant *Salmonella* in market age swine[139] did not find increases in the prevalence of resistant *Salmonella* on farms using apramycin.

Several studies have been performed to demonstrate decreases in enteropathogen shedding from food animals fed subtherapeutic antimicrobials,[189,190] specifically from swine.[140] However, an interesting early study in swine[191] demonstrated that pigs inoculated with a chlortetracycline-sensitive *Salmonella* Typhimurium were found to shed for a shorter duration if fed a diet containing chlortetracycline compared to controls, but pigs inoculated with a chlortetracycline-resistant strain were found to shed increased quantitites of organisms for a longer duration when fed a diet containing chlortetracycline. Further investigation into the effect FSAs have on the shedding duration when resistant enteropathogens are involved is needed.

5. Alternatives to in-feed antimicrobials

One of the foremost concerns in food safety today is the increase in antimicrobial resistance among bacteria from both humans and animals.[15] Feed-based subtherapeutic antimicrobials have been in use for nearly 50 years and their efficacy in both performance enhancement and disease reduction is well documented.[4-8] However, with the observed increase in antimicrobial resistance, identification of primary contributors to this increase in resistance has led to scrutiny of the use of antimicrobials in animal production.[1,12,16] This in turn has led to a call to decrease the use of antimicrobials in animal production.[169,192] Research into alternatives

to the use of antimicrobials in animal production has increased in response to recent limitations[4,193,194] and bans[13,14] on the use of antimicrobials in animal feed. The swine industry is a major consumer of FSAs,[7,28,28,171] and studies have shown increases in antimicrobial resistance from operations that use FSAs compared to those that do not.[21,26,29-31,147,172-175]

Probiotics, defined as any live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance[195] are continually being investigated for use in animal production for prophylactic disease control and improved performance. Common organisms used in probiotics include: *L. acidophilus, L. casei* ss. *casei, L. rhamnosus, L. brevis, L. lactis, Streptococcus salivarius* ss. *thermophilus, S. lactis, Enterococcus faecium, E. faecalis, Bifidobacterium bifidum, B. brevis, B. thermophilus, Bacillus subtilis, B. cereus, B. toyoi,* and *Saccharomyces cerevisiae.* These probiotic organisms can have a variety of beneficial effects on the host including reduction of bacterial enteropathogens,[38,196-199] increased growth rates,[197,200] and improved feed conversion.[197] For example, a *Streptococcus faecalis* probiotic was shown to be effective in reducing *Salmonella* and stabilizing lactic acid bacteria populations when fed to young calves and piglet.[198]

In a study comparing the effect of a zinc-bacitracin additive versus the probiotic organism *Bacillus coagulans,* piglets receiving the probiotic organism demonstrated measurable decreases in enterococci and coliform counts over time when compared to the antibiotic treated group.[201] When administered daily, the probiotic organism integrated into the enteric flora and aided in the reduction of potentially harmful coliforms. Studies evaluating *Lactobacillus* GG, a variant of *L. casei* ss. *rhamnosus,* demonstrate the ability of lactobacilli to colonize transiently, alter pH in the microenvironment, deconjugate bile acids, and reduce intestinal production of

harmful nitrogenous compounds, all of which may alter the ability of other microorganisms to survive.[202,203]

Certain lactobacillus strains have been shown to increase IgA-secreting cells in the intestinal mucosa, stimulate local release of interferon, and increase leukocyte phagocytic activity.[204,205] Recent studies confirm anti-inflammatory properties of specific probiotic strains via enhanced IL-10 production.[102] Additionally, direct beneficial effects on intestinal cell homeostasis have been identified from the use of *L. rhamnosus* GG.[111] This probiotic has been shown to inhibit cytokine-induced apoptosis *in vitro*.[206]

One study evaluating the effect of probiotic strains in an *in vitro* system called the Simulator of Human Intestinal Microbial Ecosystem (SHIME),[207] found that in addition to overall composition changes as a result of probiotic treatment, temporary increases in numbers of lactic acid bacteria were detected, and numbers of Enterobacteriaceae decreased markedly. Adherence properties of probiotic organisms have also been studied and have been identified as an important factor to consider when selecting probiotic strains.[208-211] Demonstration of adherence and protection against invading pathogens has been accomplished *in vitro*.[110] Fluorescence *In Situ* Hybridization (FISH) is a recent technology that may elucidate interactions between commensal flora and probiotics more clearly in the future.[212]

Competitive exclusion products are used to exclude enteropathogens from the lower intestinal tract by preferential colonization with beneficial bacteria indigenous to the host species. [213-216] Nurmi et al. demonstrated efficacy of competitive exclusion by preventing infection when chicks were challenged with *Salmonella* after inoculation with unmanipulated intestinal bacteria from adult birds. [36] Resistance to bacterial pathogen colonization is thought to be due largely to the anaerobic portion of the intestinal bacteria[37] and several subsequent studies

have demonstrated efficacy of CE cultures in this respect.[38-40] A review of recent literature suggests that mucosal CE products, which are derived from the normal enteric flora of a healthy animal and contain a diverse population of aerobic and anaerobic bacteria, may have a more consistent effect than probiotics alone, which are composed of relatively few bacterial species.[38,197] While animals receiving probiotics have demonstrated variable performance results,[35,41] data regarding the effect of CE on animal performance including growth is lacking. Additionally, there is little information regarding the effect(s) on antimicrobial resistant populations following use of direct-fed microbials such as probiotics and CE cultures.

Current dogma for obtaining efficacious CE cultures entails harvesting mucosal scrapings from a young healthy animal of the species to be inoculated, known to be free of pathogens and viruses; continuous flow or other propagation via culture methods under reduced or anaerobic conditions; and per oral dosing early in life and/or at times of stress such as weaning, transportation, or during clinical illness. These products have been studied most extensively in poultry which clearly demonstrate the efficacy of CE products to control *Salmonella* in both research and field conditions.[158,217-223] Studies in calves and piglets focused on exclusion of *Salmonella* and *E. coli* have demonstrated effectiveness of CE products as well.[38,40,63] While studies evaluating the efficacy of these alternatives are ongoing, to date no single intervention has demonstrated performance results comparable to those observed following use of subtherapeutic antimicrobials.

Research has shown that acidic environments may help decrease acid intolerant pathogens such as *Salmonella* and *E. coli*.[41,78,153] However, bacteria may develop acid-resistance mechanisms when exposed to acidic environments over time.[41] Other benefits of organic acid inclusion include improved performance parameters.[224] A study by Overland et. al suggests that

organic acids exert the most benefit when fed during the growth of young pigs as opposed to feeding to finishing pigs.[225]

Herbs such as garlic, clove, and peppermint have also been studied as feed supplements; however, palatability is often a challenge and the effects on performance have not been noteworthy.[35] Two recent studies investigating the effects of a seaweed extract and a plant saponin on immune function and growth performance in weaned pigs challenged with *Salmonella* also failed to produce positive results.[226,227] Overall it appears that while herbs and plant products may be useful additions to other supplements, their merit individually is questionable.

Organic acids and H_2O_2 produced by lactic acid bacteria are inhibitory against coliforms, *Salmonella*, and clostridia *in vitro.*[228] Other antimicrobial substances produced by probiotic bacteria include CO_2, diacetyl (thought to react with arginine binding protein of gram-negative bacterial, interfering with its usage), reuterin (produced by *L. reuteri*), and bacteriocins.[229] The benefits of using these antimicrobial substances *in vivo* is not yet known.

6. Antimicrobial resistance on the rise

Antimicrobial resistance is of global concern.[183,193,230,231] The apparent association between the use of FSAs in food animal production and the concomitant increase in antimicrobial drug resistance among zoonotic enteropathogens [11,12,142,142-144,167] has provided the impetus to ban the use of FSAs in food animals in Europe based soley on the precautionary principle. Of equal concern is the persistence and transfer[23-25,146,147] of antimicrobial resistance genes among commensal flora such as *E. coli*.[1,20,21,232] Although the relationship between antimicrobial resistance of a pathogen and its virulence is not fully known, it is clear that infections with resistant organisms are more difficult to treat.[143-11,18,233] The use of FSAs has

been linked to increased antimicrobial resistance in food animals.[29,68,162,164,166,167] However, the impact of veterinary antimicrobial use on human health is ill defined. Reports citing drug-resistant human infections caused by antimicrobial use in animals have increased in recent years,[143,11 233,234]but documentation to the contrary is lacking and difficult to obtain. The association between antimicrobial use and increased resistance in commensal bacteria and pathogens is relatively well established regardless of bacterial or host species. It may not be possible to quantify the contribution of antimicrobial use in the agricultural setting to the broader problem of drug resistance in humans, due to the complex nature of the development of antimicrobial resistance and large gaps in current research.

In support of a lesser impact from antimicrobial use in animals, a clinician's perspective[235] suggested that "if use of antibiotics in the veterinary field [were] to lead to development of (ultimately untreatable) infections caused by multi-resistant bacteria in man, we should be encountering multi-resistant isolates with a higher frequency in community acquired infections than in nosocomial infections." The author did acknowledge that resistant pathogens could arise from the antimicrobial use in food animals, albeit at a much lower incidence than nosocomial infections.[235] The occurrence of vancomycin-resistant enterococci (VRE) in the United States makes for an interesting point as well. Avoparcin, the drug believed responsible for the evolution of VRE has never been used in animal feed in the US, and the emergence of VRE has been associated with human use of vancomycin.[236] Interestingly, virginiamycin has been used solely as an in-feed drug for over 25 years, yet resistance to this drug in human enterococcal isolates remains approximately one percent.[9] The concern for its use in animal feeds is the potential cross-resistance to the human drug, Synercid.[13,237] Reports of VRE and/or glycopeptide-resistant enterococci from animals in the U.S. are rare.[238,239]

However, there are reports of drug-resistant human infections that have been associated with food animal antimicrobial use. Quinolone-resistant *Campylobacter* infections drew attention to the issue in Minnesota.[233] Interestingly, although greater than half the infections were likely acquired during foreign travel, concerns were raised regarding the use of fluoroquinolones in poultry production and the potential of processed poultry to serve as a reservoir of resistant *Campylobacter*. In another controversial report, a ceftriaxone-resistant *Salmonella* infection was described by Fey, et al.[143] Molecular methods were used to determine relatedness, and a connection between the patient and an outbreak of salmonellosis in calves was suggested. However, only 5 isolates were evaluated (1 from the child, 4 from cattle) and relatedness could not be definitively established.

In light of these reports, it must be remembered that the selection of antimicrobial resistance is not a simple cause and effect relationship. In considering some of the variables for animals alone, in one study, R-factor transfer in sheep was greatly enhanced by stress (starvation) of the animal and persisted in some animals for months in absence of any antimicrobial treatment of the animals.[240,241] Environmental factors such as cold stress, overcrowding, and poor sanitation[242] have a significant effect not only on the selection for antimicrobial resistance but its persistence as well.[23] In addition to use of antimicrobials in animal agriculture, practices not related to animal agriculture that also impact the development of resistance should be scrutinized as well. Heavy metals and certain disinfectants are also culprits in selecting for antimicrobial resistance.[243] Consideration must be given to the human impact on antimicrobial resistance via use of disinfectants and antimicrobial agents in soaps, toothpaste, and other products, as well as application of micronutrients and fertilizers in agriculture.

Resolution of the resistance problem can only arise from a "big picture" approach, addressing the many factors involved in the use of antimicrobials, development and selection of resistance in both animals and humans. Removal of antimicrobials in animal feed may have some effect on resistance as seen in Denmark,[13] but removal alone is not the final answer. While the abolishment of FSAs in Denmark did result in a reduction of resistance among food animals,[13] increases in disease among animals has resulted in a rise in the therapeutic use of antimicrobials.[244] A reasonable solution will take into consideration that the development of antimicrobial resistance involves a complex web of events and is a natural consequence of use followed by the inherent adaptation ability of bacterial cells to resist killing.

To provide further information regarding the effect of specific FSAs and PCE culture on the commensal *E. coli* population in growing pigs, the following studies were based on two primary objectives: 1) To determine the effect of three feed-based subtherapeutic antimicrobials (apramycin, chlortetracycline, and carbadox) and 2) the effect of porcine-derived mucosal competitive exclusion culture on both the antimicrobial susceptibility profiles of commensal *E. coli* populations and animal performance in growing pigs.

CHAPTER 3

EFFECT OF FEED-BASED SUBTHERAPEUTIC ANTIMICROBIALS ON *E. COLI* FROM GROWING PIGLETS

Introduction

Feed-based subtherapeutic antimicrobials (FSAs) have been in use for nearly 50 years and their efficacy is well documented.[4-8] Continued concerns regarding an apparent association between the use of FSAs in food animal production and a concomitant increase in antimicrobial drug resistance among zoonotic enteropathogens have provided the impetus to ban the use of FSAs in animal feeds. [9-12] In Europe, bans based on the precautionary principle against FSAs such as avoparcin and virginiamycin, are already in place.[13-15] The potential benefits versus risks of FSA use are currently being debated in the United States.[4,16,17] Recent reports suggest that antimicrobial resistant foodborne infections may be associated with higher morbidity compared to infection with susceptible pathogens.[a, 18, 19] However, the correlation between use of FSAs and the impact on human health remains unclear.

Commensal bacteria, such as *Escherichia coli* (*E. coli*), are thought to be reservoirs of resistance genes, capable of transferring genes to foodborne and other zoonotic pathogens.[1,9,20,21] *Escherichia coli* strains may be commensal or pathogenic. They are found in the digestive tract of most animals and are ubiquitous in the environment. The occurrence and persistence of antimicrobial resistance in these bacteria has been documented,[22-26] and a recent report suggests that any *E. coli* strain acquiring the appropriate virulence factors may become pathogenic.[27]

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[a] Personal communication: Steve Carlson PhD, USDA-ARS College Station, Texas.

The swine industry is a major consumer of FSAs,[7,28] and studies have shown increases in antimicrobial resistance in enteric bacteria isolated from operations that use FSAs.[29,30,31] Epidemiologic studies suggest that enteric flora from pigs often demonstrate resistance to antimicrobials which have been in use in both human and animal medicine for over 40 years especially tetracycline, streptomycin, and sulfonamides.[32-34] The present study was designed to determine the effect of three FSAs (apramycin, carbadox, and tetracycline) on the antimicrobial susceptibility of commensal *E. coli* in growing piglets.

Materials and Methods

Animals and facility. Crossbred sows were obtained from the University of Georgia's (UGA) Swine Center for all trials. All breeding was accomplished via artificial insemination. One week prior to farrowing, sows were moved to UGA's Animal and Dairy Science Complex (ADSC) farrowing unit and randomly allotted by parity to treatment groups. For Trial 1, 7 of 8 sows were Hampshire-Large White (H-LW) crosses bred to Large White boars. The last sow was a Landrace-Welsh cross. For Trial 2, 5 of 7 sows were H-LW bred as above, while the remaining two were Landrace-Hampshire crosses. In Trial 3, all sows were Yorkshire-Hampshire-Large White crosses bred to a Dru terminal-cross boar (French Musculor and American Duroc) (Appendix A). Piglets were cross-fostered within 72 hours post-farrowing as needed to adjust litter size and ensure maximum survival. The facilities at ADSC were environmentally controlled and provided *ad libitum* water for sows and piglets (Appendix B). Lighting was automated to provide a 16-hour photoperiod daily. At all times during the farrowing, lactation, and nursery phases, pigs in both groups were housed within the same rooms, but on separate sides of 6 foot concrete aisles. The pull-plug pit systems below the pens were also separate and each was drained and refilled weekly. Care was exercised to handle and

sample FSA-free control pigs before handling those fed FSAs. At sampling, coveralls, boot covers, and gloves were donned upon entry to the unit then changed between groups. At weaning, piglets were moved to the ADSC pig growth room by litter and sorted by weight and sex into pens of 2 to 4 piglets per pen (3 ft x 6 ft). At approximately 11 weeks of age, piglets were individually tagged and returned to the Swine Center where space limitations required commingling of both groups regardless og control or treatment status.

Experimental design. Three replicate trials were conducted using different sows. Each trial was composed of 2 groups (up to 45 piglets per group). In the control group, piglets were fed diets without FSAs (antimicrobial-free). For the treatment group, piglets were fed diets with FSAs. Sows and piglets were sampled at regular intervals for fecal *E. coli*, and recovery of 12 *E. coli* isolates per animal was attempted at each sampling. The sampling and diet schedule is shown in Table 3-1. Animals were managed per standard production practice guidelines established at the UGA Swine Center including diets and duration of feeding.[b] All diets were appropriate to age and phase of growth or gestation/lactation and formulated to meet or exceed National Research Council requirements (Appendices C, D).[245] All sows were fed antimicrobial-free diets starting at ~100 days of age. Gestation and lactation diets were antimicrobial-free, and sows were hand-fed for maximal feed intake (20lb-plus per day) at peak lactation. For FSA-containing diets, the antimicrobials used and the amount and duration of feeding is shown in Table 3-2. Piglet diets were identical in formulation and physical form/processing except for the addition of FSAs for the treatment group. The prestarter diet (S1) was fed as a creep feed starting on day 7. At weaning (Day 28 - Phase 1) 5lb/pig of the S1 diet was dispensed and consumed in approximately 6 days. On Day 34 (Phase 2) 15lb/pig of the S2 was dispensed and consumed in about 9 days. In Phases 3 (S3 diet) and 4 (G1 diet), the diets were fed *ad libitum*.

Phase 4 could not be concluded in the grower facility due to space limitations, and was restricted to 14 days before all pigs were returned to the Swine Center.

Performance. Piglets were weighed at birth, and on days 21, 28 (weaning), 34, 43, 61, 75, and 171 prior to market (Table 3-1). Performance measurements included adjusted 21-day weights,[246] average daily gains, daily feed intake, and feed efficiencies for Phases 1-4 (Days 28-75) only. Prior to market, pigs were weighed to calculate adjusted days to 250 lb and adjusted backfat measurements.[246] Evaluation of health status, including screening for parasites and detection of infectious diseases, was also performed as needed.[c]

Bacteriology. The farrowing and grower facilities were sampled for *Salmonella* and *E. coli* prior to each use. Ten to 15 drag swabs pre-moistened with PBS were taken per site from crate dividers, mats, feeders, waterers, doors and aisleways and placed in sterile Whirl-pak™ bags prior to the addition of 20mls of BHI broth (Difco, Detroit, Michigan). Feces from sows were cultured for *Salmonella* and *E. coli* 7 days pre- and 28 days post-farrowing. Post-farrowing, fecal samples were taken regularly from all piglets (Table 3-2) for culture of *E. coli* only. After piglets returned to the Swine Center, fecal samples were taken randomly from half of barrows and gilts in each group at approximately 3-week intervals until the animals reached market weight.

Salmonella isolation was performed as follows (Appendix E); 1 gram of feces was diluted in 9mls phosphate buffered saline (PBS) and 100ul transferred to 9.9mls each of tetrathionate broth and GN-Hanja broth (Difco, Detroit, Michigan). The GN-Hanja broth was incubated overnight at 37°C, and the tetrathionate broth was incubated for 48 hours at 37°C. Approximately 100ul of each was transferred to Rapport Vassiliadis Broth R-10 (BD

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[b] UGA Swine Center Operations Manual, ADSC Swine Center, Athens, GA, 30602
[c] Athens Diagnostic Laboratory, University of Georgia, Athens, GA 30602

Microbiology Systems, Sparks, Maryland), and incubated at 37°C overnight. Each RV subculture was plated to both xylose-lysine-tergitol 4 agar (XLT-4, BD Microbiology Systems) and brilliant green agar with sulfadiazine (Difco) and incubated overnight at 37°C. Presumptive positive colonies were confirmed using TSI and LIA slants. Positive colonies were serogrouped using *Salmonella* O-antiserum (BD Microbiology Systems) and sent to the National Veterinary Services Laboratories in Ames, IA for serotyping. Environmental samples were processed for *Salmonella* by adding 1ml of the enriched BHI broth to 9mls PBS, and transferring 100ul to 9.9mls each of tetrathionate broth and GN-Hanja broth and proceeding as above.

Escherichia coli (Appendix E) was isolated by diluting 1 gram of feces in 9mls PBS. Approximately 100ul of sample was direct plated to Chromagar® ECC agar (Hardy Diagnostics, Santa Maria, California) and incubated 18-24 hours at 42°C. For piglets, one swab (used through Day 14 for all pigs and for small piglets to Day 21) or fecal loop from each piglet was diluted in 3mls PBS then direct plated to Chromagar® ECC agar as described above. For environmental samples, 100ul of BHI broth from the drag swab was direct plated to Chromagar® ECC agar as described above, and after overnight enrichment at 37°C, another 100ul was plated to Chromagar® ECC agar. Recovery of 12 colonies per sow or piglet was attempted, and blue-green colonies were preferentially selected (Appendix F).[247] For plates that did not contain typical blue-green colonies, non-typical colonies were selected and tested to confirm they were *E. coli* using biochemical methods such as the BBL™ Crystal™ Enteric/Nonfermenter ID Kit (BD Diagnostic Systems), the API-20E® Enteric Identification System (bioMérieux, Inc., Hazelwood, Missouri) or Vitek (bioMérieux, Inc.).

Antimicrobial susceptibility. Phenotypic antimicrobial susceptibility screening of all *E. coli* isolates was accomplished using a replica-plating method previously described.[248,249] The

screening included tetracycline and apramycin (FSAs used in the feed, Table 3-1), and gentamicin. Gentamicin is not used in animal feeds. However, it is representative of the aminoglycoside class to which apramycin belongs and was included to detect differences in resistance mechanisms for the aminoglycosides.[180] The National Committee for Clinical Laboratory Standards interpretation criteria for breakpoints was used for tetracycline (16ug/ml). For apramycin (32ug/ml) and gentamicin (16ug/ml), breakpoints used by the National Antimicrobial Resistance Monitoring System[d] (NARMS) were followed.[250] Colonies to be tested were transferred to a gridded EC Medium with MUG plate (Difco) and incubated overnight at 37°C. The colonies were inoculated onto a sterile felt, then replicated onto 6 Mueller Hinton II agar plates (BD Microbiology Systems) containing the antimicrobials to be tested at doubling dilutions, ending with a final antimicrobial-free plate to ensure adequate and even transfer of colonies (Appendix G). All plates were incubated overnight at 37°C. One person was responsible for reading all plates to minimize subjectivity. Colonies were subjectively scored as 0 (no growth) and 1 (visible colony). To validate the scoring system, subjective scores were compared to MIC values using Sensititre® results from selected isolates (Appendix H).

Additional antimicrobial susceptibility testing was performed on environmental isolates, *Salmonella* isolates, and selected *E. coli* isolates from piglets in each group and trial using a 96-well custom made panel (Sensititre,® Trek Diagnostic Systems, Inc., Cleveland, Ohio) per manufacturers' instructions. Disk diffusion on Mueller Hinton II agar plates was also employed to test 486 isolates from all piglets in Trial 1 from Day 7, Day 28 (weaning), and Day 61 for

[d] NARMS web page available at: http://www.fda.gov/cvm/index/narms/narms_pg.html

carbadox resistance.[184] Growth inhibition zones of <10mm were interpreted as resistant to carbadox. All isolates were stored in Mueller Hinton Broth (Difco) with 10% glycerol at –90°C.

Statistical Analysis. Dichotomous antimicrobial resistance data from the replica-plating was analyzed using regression analysis of count data for repeated measures.[251] Results from piglets receiving FSAs were compared to controls using least square means obtained from the general linear model (GLM) of the Statistical Analysis System procedure (SAS Inst. Inc., Cary, North Carolina). Resistance data for piglets obtained from the Sensititre® testing was evaluated by assigning dichotomous values of sensitive (0) versus resistant (1), and data pertaining to resistance profiles from replica-plating were analyzed using Chi-square analysis. Performance data were also analyzed using GLM procedures from SAS.

Results

Piglets in all trials were predominantly healthy, although minor individual health problems were encountered including septic arthritis and abscesses in pigs from both groups. Pigs requiring antimicrobial treatment were removed from the study. During Trial 1, one piglet died at weaning (caught under feeder), and one piglet apparently died due to gastroenteritis (no known etiology, littermates unaffected). No death losses occurred in Trials 2 and 3; however, during Trial 2, 1 piglet was removed after weaning due to severe respiratory condition that required medical treatment and the penmate was also removed. Additionally, the majority of piglets in the control group of Trial 2 suffered rotaviral diarrhea at weaning. However, no antimicrobial or other treatment was given and all pigs recovered within ~7 days. For any group exceeding 45 piglets at weaning, random piglets were selected for return to the Swine Center due to space limitations in the ADSC.

During Trial 2, a sow in the control group developed cystitis post-farrowing that required medical treatment with procaine penicillin, dexamethasone, vitamin B-complex, and cimetidine. The sow was retained in the study throughout and after treatment, and monitored closely. Because of a significant decrease in lactation, her piglets were given supplemental milk replacer[e] that contained oxytetracycline and neomycin base 140g/ton each from 10 days of age through weaning. *Escherichia coli* isolated from her piglets did not show increased resistance to the antimicrobials tested, despite the sow's treatment and the antimicrobials in the milk replacer.

Animal performance was significantly affected by FSA use during Phases 3 and 4 across all trials of the study. Differences in average daily gains in Phases 3 and 4, $P < 0.0001$ (Figure 3-1), and in feed efficiency during Phase 4, $P < 0.001$ (Figure 3-2) demonstrate that the FSA piglets had higher gains and improved feed efficiency. Daily feed intakes were also significantly higher for the piglets fed FSAs, $P < 0.0001$ (Appendix I). Since trials were grouped for analysis, the significant difference between groups for feed efficiency during Phase 1 was likely due to decreased feed intake by piglets from Trial 2 that suffered rotaviral infection following weaning. For measurements calculated prior to market (adjusted-days to 250lbs and backfat), no differences between the treatment groups were detected.

For all trials, *E. coli* were recovered from 98.9% (1981/2004) of sow samples, 98.8% (25235/25542) of piglet samples, and 42.0% (29/69) of environmental samples. For sows, *Salmonella* was isolated only during Trial 2. During Trial 2, 3 of 7 sows tested positive (2.5% or 4/161 samples). All other samples from Trials 1 and 3 (sows and environmental samples) were negative for *Salmonella*.

Salmonella isolates were pansensitive by Sensititre®. Of the 4 isolates recovered in Trial 2, 2 were from sows in the control group, and 2 were from a sow in the treatment group on

[e] Rescue Baby Pig Milk Replacer, Akey, Inc, Lewisburg, OH 45338

different sampling days. *Salmonella* Cerro (group K) was isolated from a control sow upon arrival at the ADSC. When piglets reached 21 days, two more *Salmonella* isolates were identified as untypable *Salmonella* 42:Z4, Z23 from another control sow, and *S.* Newington (group E2) from a sow in the treatment group. At weaning a final isolate was identified, from the same sow originally positive for *S.* Newington in the treatment group, however, a different serotype, *S.* Soerenga (group N) was isolated. Neither sows nor piglets exhibited clinical signs of salmonellosis during any of the trials.

Twenty-two to 25 environmental samples were taken from the farrowing and grower facilities prior to stocking in each trial. *E. coli* isolates were recovered only from Trials 2 (n=17) and 3 (n=12). Trial 1 was the first group of pigs into the new facility at ADSC. Antimicrobial susceptibility profiles from environmental *E. coli* (Figure 3-3) exhibited resistance to ampicillin, kanamycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim sulfamethoxazole. Antimicrobial resistance in enterococci isolated from the environment is shown in Appendix J.

The replica-plating results from piglets were compared to selected isolates tested using Sensititre® (Appendix H). Sensitivity of the replica-plating method compared to Sensititre® results (correct identification of resistant isolates) was 96.2% for the aminoglycosides (apramycin and gentamicin) and 99.1% for tetracycline. Specificity (correct identification of sensitive isolates) was 91.4% for the aminoglycosides, and 92.5% for tetracycline.

In total 27,049 *E. coli* isolates from 23 sows and 204 piglets were screened (Trial 1: n=7,598, Trial 2: n=8,886, Trial 3: n=10,732). Differences between sow groups were not significant in any trial for the 3 antimicrobials tested. For piglets, significant differences were observed in the proportion of: 1) pansusceptible *E. coli* isolates, 2) those resistant to both apramycin and gentamicin, and 3) isolates resistant to all 3 antimicrobials tested (tetracycline,

apramycin and gentamicin), $P < 0.0001$ (Table 3-3). The proportion of isolates resistant to tetracycline was highly variable ranging from 10% to 92% and 34% to 99% of isolates for sows and piglets, respectively (Figures 3-4 and 3-5). Tetracycline resistance increased in the FSA treated pigs on Days 34, 61 and 75. A significant increase in the percentage of resistant isolates was detected in piglets during feeding of subtherapeutic tetracycline (Day 75 - Phase 4) when compared to controls ($P < 0.0001$). Apramycin resistance in *E. coli* from sows was relatively low with group averages from 0 to 6% (Appendix K). For piglets, apramycin resistance in *E. coli* is shown in Figure 3-6. *Escherichia coli* from FSA-treated piglets overall trials demonstrated significant increases in resistance to both apramycin and gentamicin ($P < 0.0001$). In Trials 1 and 3 only, resistance to apramycin was higher in piglets fed subtherapeutic apramycin (Phase 1), particularly on Days 28, 34, and 43, then declined with its removal to less than 2% and 12%, respectively, during Phase 4 (data not shown). However, piglets fed subtherapeutic antimicrobials in Trial 2 did not exhibit increased resistance to apramycin or gentamicin compared to controls. Gentamicin resistance paralleled apramycin resistance for both sows and piglets, respectively (data not shown). After piglets were returned to the Swine Center where treatment groups were commingled, the significant differences in antimicrobial resistance between treatment groups did not persist (Figure 3-7). However, on Day 118, tetracycline resistance was significantly higher in the FSA group pigs ($P < 0.05$), and on Day 171, apramycin resistance was higher in the control group piglets ($P < 0.05$). Although there were differences observed between groups on these specific sampling days there remained no significant differences, over all, for the commingled groups.

Antimicrobial susceptibility results from selected isolates tested using the Sensititre® system (n=1486) indicated that some *E. coli* isolates were also resistant to amoxicillin-clavulanic

acid (1.5%), ampicillin (17.6%), cephalothin (2.0%), kanamycin (12.7%), streptomycin (27.8%), sulfamethoxazole (20.3%), and trimethoprim sulfamethoxazole (4.2%) (Table 3-4). In addition to tetracycline, apramycin, and gentamicin as previously shown, over all 3 trials, significant differences between groups were only detected for tetracycline, gentamicin, and kanamycin ($P < 0.01$). Additional information regarding antibiogram patterns exhibited by 3 or more isolates are presented in Appendix L (n=1448 or 97.4% of total isolates tested). Resistance to carbadox was not detected in either group of Trial 1 (486 isolates tested from Days 7-61) and no further testing of isolates was pursued in subsequent trials (Appendix M).

Discussion

The reported benefit of FSAs on growth promotion is primarily due to improved feed efficiency, and has been demonstrated in previous field studies.[7,8,252] Despite a relatively small sample size, significant differences in animal performance were detected in the present study. The FSA-fed piglets outperformed piglets in the control group in Phases 3 and 4. These results suggest that FSAs may not affect performance uniformly through the grow-out period. Additionally, this study did not evaluate FSAs for their ability to prevent or minimize disease. Individual health problems were minimal and were noted in all trials as described above.

The sensitivity and specificity of the replica-plating method provides an evaluation of the reliability of results. This method detects resistant isolates when present (sensitivity) 99-100% of the time for tetracycline and gentamicin, and 83.3% for apramycin. As identification of resistance is the primary objective of this study, a high degree of sensitivity was desired. While the sensitivity estimation for apramycin was lower, fewer isolates were tested on Sensititre® for resistance to apramycin (n=165) versus gentamicin and tetracycline (n=580) due to changes made to the customized plate used. Additionally, isolates resistant to apramycin were also

resistant to gentamicin; therefore, overall aminoglycoside sensitivity was calculated (96.2%). For specificity, the replica-plating method detects 91.4-92.5% of sensitive isolates correctly. As a result, this study likely overestimated resistant isolates approximately 7% to 9% of the time during the initial screening.

Subtherapeutic antimicrobials have been routinely used in animal feeds for decades. However, their potential role in initiating the development and persistence of antimicrobial resistance has increased concerns regarding continued use of FSAs. While several studies have demonstrated decreases in *Salmonella* shedding from food animals fed subtherapeutic antimicrobials,[140,189,190] these studies did not evaluate effects on antimicrobial resistance in bacterial populations.

Tetracycline has been in use in animal production since the 1950s, and resistance among commensal fecal organisms of food animals appears common regardless of antimicrobial treatment.[31,162,174] Removal of the antimicrobial pressure can have a significant effect, as demonstrated by a nearly 50% drop in resistance to tetracycline in enteric flora of pigs from a closed herd 126 months after removal of all antimicrobials.[173] In this study, it is interesting to note the high percentage of isolates resistant to tetracycline in the control group which appear to persist in the absence of tetracycline use. Resistance to tetracycline in isolates from sows of both groups decreased from pre-farrowing (~70%) to weaning (~34%). This suggests that the initial resistance level observed may have been elevated due to the stress of farrowing. However, an increase was seen in resistance to tetracycline with FSA use suggesting that tetracycline resistant populations can expand with FSA use as observed during Phase 4. The presence of subtherapeutic levels of tetracycline in the feed during Phase 4 may have allowed expansion of tetracycline-resistant isolates as they out-competed the tetracycline-sensitive isolates. After pigs

returned to the Swine Center, tetracycline was removed from the diet at Day 118, and resistance persisted through Day 151. Resistance to tetracycline in *E. coli* was present in both groups and no difference was detected between treatment groups during this time. Tetracycline resistance did decrease in both groups by Day 171, but did not return to levels observed at the ADSC. As tetracycline is often used in diets for finishing pigs, further investigation as to the production benefit versus impact in further increasing the resistant *E. coli* population versus human health impacts are warranted. A recent epidemiologic study suggests that subtherapeutic antimicrobial use is most beneficial during the nursery phase, and that use of antimicrobials during the finishing phase be limited to therapeutic use in confirmed infections.[253]

Use of apramycin appears to result in significant increases in resistance among enteric bacteria that rapidly declines to a low level of resistance with its removal. This finding is in agreement with previous studies that demonstrate low levels of apramycin resistance among *E. coli* from market age swine fed apramycin as piglets.[139,254] Although apramycin increases the resistant population of *E. coli*, the rapid decline following its removal suggests that it has little effect on the maintenance of resistant populations and may be useful in prevention of disease when used for short duration. Further, this rapid increase, then decrease, following use of apramycin is suggestive of a specific selective pressure suppressing apramycin-sensitive *E. coli*, and allowing for the expansion of apramycin-resistant *E. coli*. However, once the selective pressure is removed, subsequent repopulation of sensitive isolates occurs.

As expected, results for gentamicin resistance paralleled that of apramycin in the present study, suggesting that resistance to drugs within the same class is likely due to a common resistance mechanism. Apramycin resistance is mediated by only one enzyme, but other aminoglycosides such as gentamicin are mediated by up to 11 different enzymes.[180] The

resistance to gentamicin declined along with the apramycin resistance and suggests a potential for the development of persistent cross resistance for these two antimicrobials.

While the present and previous studies have shown a positive correlation between the feeding of subtherapeutic antimicrobials and increased resistance in commensal *E. coli* organisms,[29-31,186] further understanding of individual antimicrobials and their effect on commensal populations of bacteria is needed. During Trial 2 of the present study, increased resistance to apramycin and gentamicin was not observed. No differences in management, diet, or sows could be determined and it is not known why the *E. coli* populations in these pigs did not respond to the selective pressure of apramycin. Additionally, *E. coli* from the piglets in Trial 2 from the sow that developed cystitis post-farrowing did not show increased resistance to the antimicrobials tested, despite the sow's treatment and the antimicrobials contained in the milk replacer which was direct-fed to the piglets.

Aptly stated by Dr. A.O. Summers,[255] "…propagation of resistance is an ecological problem." Therefore, the key to understanding this problem requires acknowledgment of the complex network of factors involved. Antimicrobial use alone does not necessarily equate to the development of resistance and the entire ecology of the production environment must be considered. The remarkable lack of apramycin resistance in *E. coli* isolates from Trial 2 in FSA-treated piglets and from control piglets receiving the FSA-containing supplemental milk provide excellent examples.

In a recent study, the effect of different antimicrobial regimes was evaluated in swine.[187] Rotation with similar classes of antimicrobials resulted in development of the greatest percent resistance in *E. coli* to the antimicrobial tested. Conversely, pulse-dosing resulted in the least amount of resistance. This information can be put to practical use by synchronizing dosing

regimes, and type of antimicrobial chosen, to provide the greatest impact on arresting disease while having minimal effect on the development of resistance.

Carbadox has been banned in Europe and Canada because of potential teratogenic and carcinogenic effects.[182,183] Resistance to carbadox is uncommon as shown in the present study and previous reports.[68,139] However, consideration should be given to the antimicrobial testing method used for carbadox. Isolates in this study were tested using an aerobic disk diffusion method. One report suggests that anaerobic testing provides superior results and should be further investigated.[184]

Implications: The development of antimicrobial resistance in zoonotic and commensal bacteria from food animals has resulted in complete cessation of subtherapeutic antimicrobial use in Europe, and proposals in the U.S. to end the use of subtherapeutic antimicrobials in animal feeds. Although baseline resistance levels to tetracycline were relatively high in the present study, increased resistance was observed following tetracycline use in feed. Conversely, apramycin resistance rapidly increased then decreased to baseline levels following its use. These data suggest that some antimicrobials, such as apramycin, may not lead to the development of persistently resistant populations and may therefore be suitable for continued use. Understanding the effects of different classes of antimicrobials on the antimicrobial resistance of commensal flora will assist in making science-based decisions concerning the future use of feed-based subtherapeutic antimicrobials.

Table 3-1. Antimicrobial content of diets fed to treatment piglets (Group 1 only), Trials 1-3. Group 2 (controls) were fed identical diets without antimicrobials on the same schedule.

Phase	Diet	Antimicrobial in diet	Product name	gm/lb in feed	Amount or duration fed	Comments
Phase 1 (~6 days)	**S1** (starter 1)	Apramycin sulfate	Apralan 7.5[a] (7.5g active ingredient/lb)	0.075	5lb per pig at weaning	Also creep fed starting on Day 7
Phase 2 (~9 days)	**S2** (starter2)	Carbadox	Mecadox 2.5[b] (2.5g active ingredient/lb)	0.025	~15lb per pig	--
Phase 3 (18 days)	**S3** (starter 3)	Carbadox	Mecadox 2.5[b] (2.5g active ingredient/lb)	0.025	~18 days *ad libitum*	--
Phase 4 (14 days)	**G1** (grower 1)	Chlortetracycline hydrochloride	Aureomycin 50[b] (50g active ingredient/lb)	0.05	14 *days* ad *libitum*	G1 continued on farm for 3 weeks (~5 weeks total)
--	**G2** (grower 2)	None	--	--	~40 days *ad libitum*	Diet fed on farm
--	**F1** (finisher 1)	None	--	--	*ad libitum* until market	Diet fed on farm

[a] Farmland Industries, Kansas City, MO 64195
[b] Akey, Inc., Lewisburg, OH 45338

Table 3-2. Sampling schedule for piglets.

Day	Diet: Sows	Diet: Piglets	Animals sampled	Measurements	Other procedures
-7	Gestation diet	--	Sows prior to farrowing	Birth weight	Process within 24 hrs of birth: ear notch for identification, dock tails, clip milk teeth, 1ml iron[a] IM
7	Lactation diet	Creep feed **S1** diet	Piglets (~7 days of age) and sows	--	Vaccinate[b] piglets IM, castrate males, and start creep feed
14	Lactation diet	Creep feed **S1** diet	Piglets and sows	--	Piglets ~14 days of age; vaccinate[c] sows IM
21	Lactation diet	Creep feed **S1** diet	Piglets and sows	Weight at 21 days	--
28	Lactation diet	Creep feed **S1** diet	Piglets and last sample for sows	Weight at weaning	Wean piglets at ~28 days and re-vaccinate; sows return to farm
Phase 1 Day 34	--	**S1** diet 5lbs/pig	Piglets	Weight at end of S1 diet	--
Phase 2 Day 43	--	**S2** diet 15lbs/pig	Piglets	Weight at end of S2 diet	--
Phase 3 Day 61	--	**S3** diet *ad libitum*	Piglets	Weight at end of S3 diet	Weigh back remaining starter 3 diet and remove
Phase 4 Day 75	--	**G1** diet *ad libitum*	Piglets	Weight prior to return to farm	Tag pigs prior to returning to farm (continue grower feed)
94	--	**G1** diet *ad libitum*	Random sampling	--	Piglets mixed on farm
118	--	**G2** diet *ad libitum*	Random sampling	--	--
151	--	**F1** diet *ad libitum*	Random sampling	--	--
171	--	**F1** diet *ad libitum*	All pigs from study	Market weight	Measure back fat

[a] Ferrodex 100 Iron hydrogenated dextran 100mg/ml; AgriLabs, St. Joseph, MO 64503
[b] ProSystem® B*P*M*E *Bordetella bronchiseptica*, *Pasteurella multocida*, *Mycoplasma hyopneumoniae* bacterin, *Erysipelothrix rhusiopathiae* bacterin toxoid (U.S. Patent 5,338,543); Intervet, Inc., Millsboro, DE 19966
[c] Breed Sow 7 Parvovirus vaccine – killed*, Erysipelothrix rhusiopathiae; Leptospira canicola, grippotyphosa, hardjo, icterohemorrhagica, pomona* bacterin toxoid, AgriLabs, St. Joseph, MO 64503

Table 3-3 **Piglets:** replica-plate antimicrobial susceptibility profile results for *E. coli* isolates, Trials 1-3.
Significant differences detected between groups noted by asterisk, *P < 0.0001.
NON=isolates from control piglets, FSA=isolates from FSA-treated piglets; n=19,704.

Antimicrobial	***Percent resistance***			
	Control piglets		FSA-treated piglets	
	# isolates	(%)	# isolates	(%)
Pansensitive	3907	38.0	2475	*26.3
Tetracycline	5875	57.2	5334	56.6
Apramycin + gentamicin	38	0.4	472	*5.0
Tetracycline + apramycin + gentamicin	430	4.2	1094	*11.6

Table 3-4 **Piglets**: additional antimicrobial susceptibility performed on selected isolates using Sensititre®, Trials 1-3.
Significant differences noted by asterisk, *P < 0.01.
NON=isolates from control piglets, FSA=isolates from FSA-treated piglets; n=1,486.

Antimicrobial	***Percent resistance***			
	Control piglets		FSA-treated piglets	
	# isolates	(%)	# isolates	(%)
Amoxicillin-Clavulanic Acid	1	0.1	21	3.0
Ampicillin	139	18.7	116	16.5
Cephalothin	6	0.8	23	3.3
Gentamicin	11	1.5	51	*7.3
Kanamycin	68	9.1	116	*16.5
Streptomycin	192	25.8	210	29.9
Sulfamethoxazole	149	20.0	145	20.6
Tetracycline	462	62.0	486	*69.1
Trimethoprim Sulfamethoxazole	21	2.8	40	5.7

Figure 3-1. Average daily gains (ADG) for piglets in Trials 1-3.
Statistical significance: FSA-treated piglets had significantly higher ADGs in Phases 3 and 4 (noted by asterisk) *P < 0.0001.
Error bars represent 1 standard error derived from the statistical model.
Groups: NON=control piglets, FSA=FSA-treated piglets; n=204.

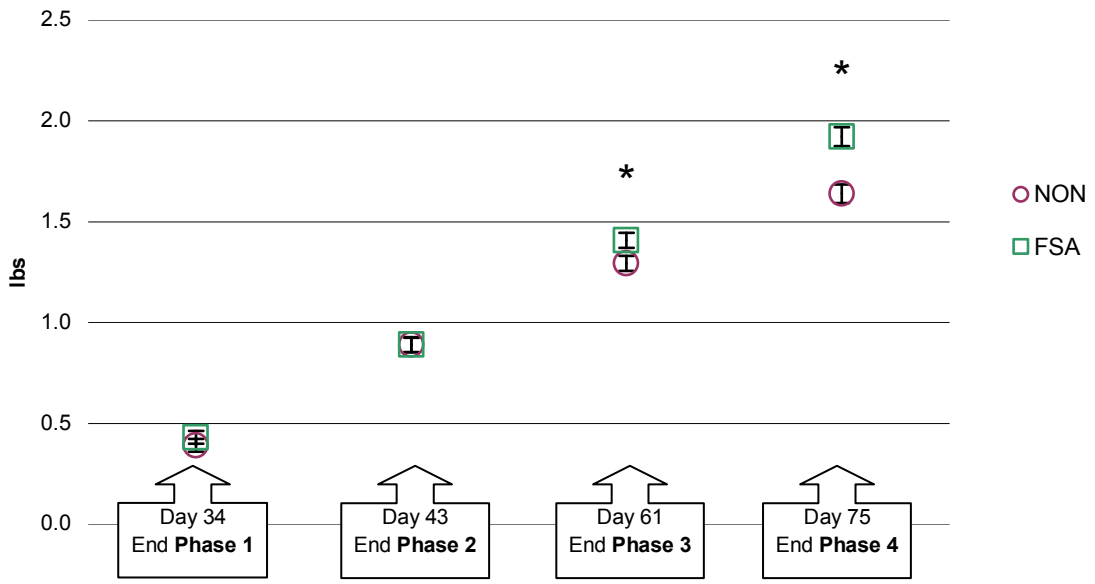


Figure 3-2. Feed efficiencies (FE) for piglets in Trials 1-3.
Statistical significance: FSA-treated piglets had significantly improved FE in Phases 1 and 4 (noted by asterisk) *P < 0.001.
Error bars represent 1 standard error derived from the statistical model.
Note: The difference identified during Phase 1 likely due to the rotaviral diarrhea that occurred in control piglets following weaning during the second trial.
Groups: NON=control piglets, FSA=FSA-treated piglets; n=204.

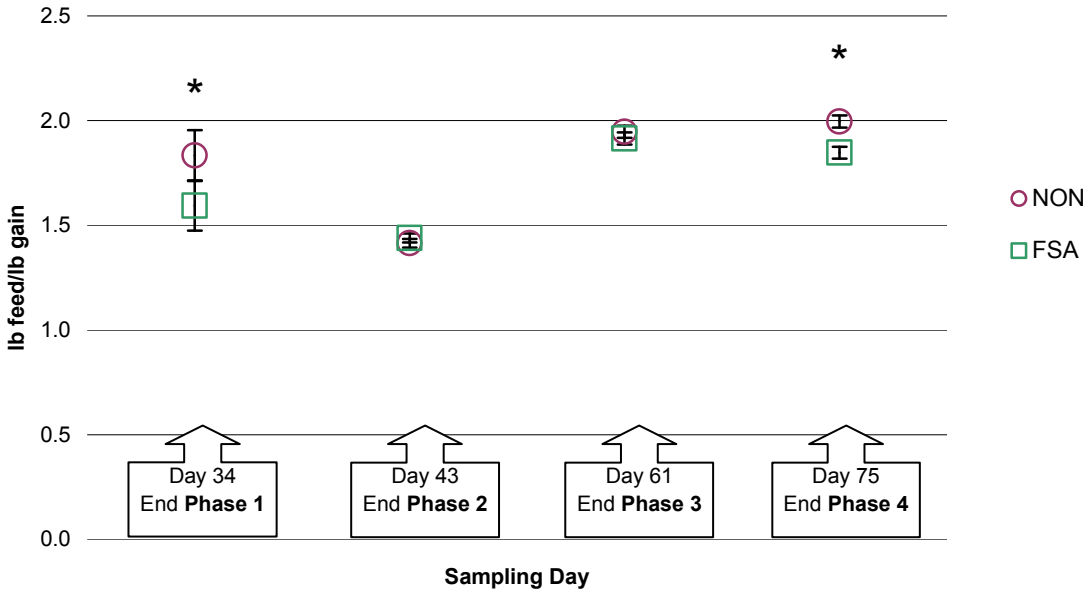
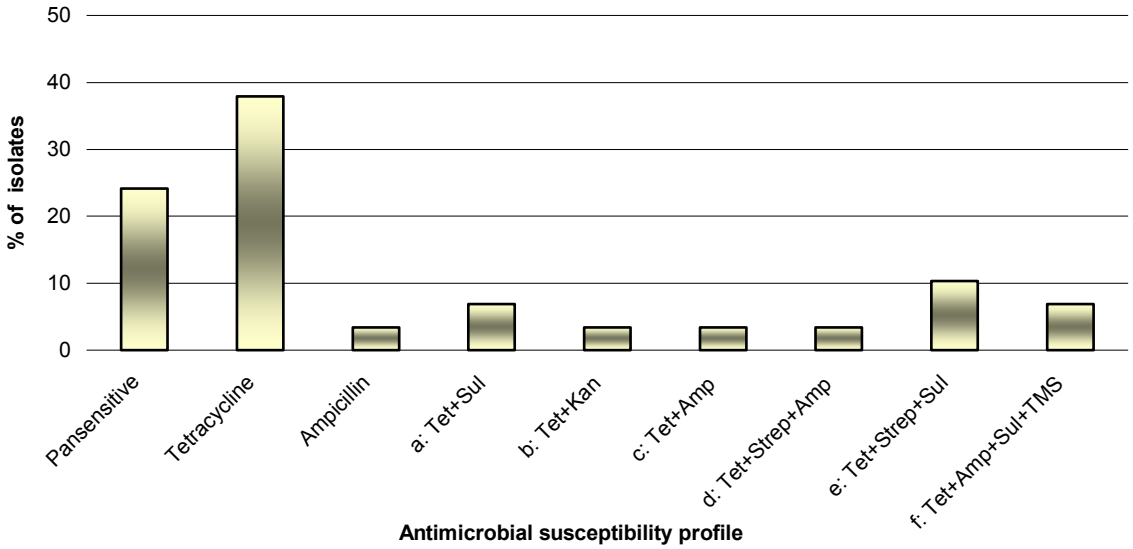


Figure 3-3. Antimicrobial susceptibility profiles of *E. coli* isolates from environmental samples; n=29.
Note: Scale to 50% only.



a: tetracycline and sulfamethoxazole
b: tetracycline,and kanamycin
c: tetracycline and ampicillin
d: tetracycline, streptomycin, and ampicillin
e: tetracycline, streptomycin, and sulfamethoxazole
f: tetracycline, ampicilllin, sulfamethoxazole, and trimethoprim sulfamethoxazole

Figure 3-4. **Sows**: total percent tetracycline resistance at 16ug/ml in *E. coli* isolates, Trials 1-3.
Statistical significance: No significant difference between groups over all samples collectively. Sows of FSA-treated piglets had significantly increased tetracycline resistance prefarrow and at Day 21(noted by asterisk), *P < 0.05.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from sows of control piglets, FSA=isolates from sows of FSA-treated piglets; n=1,981.

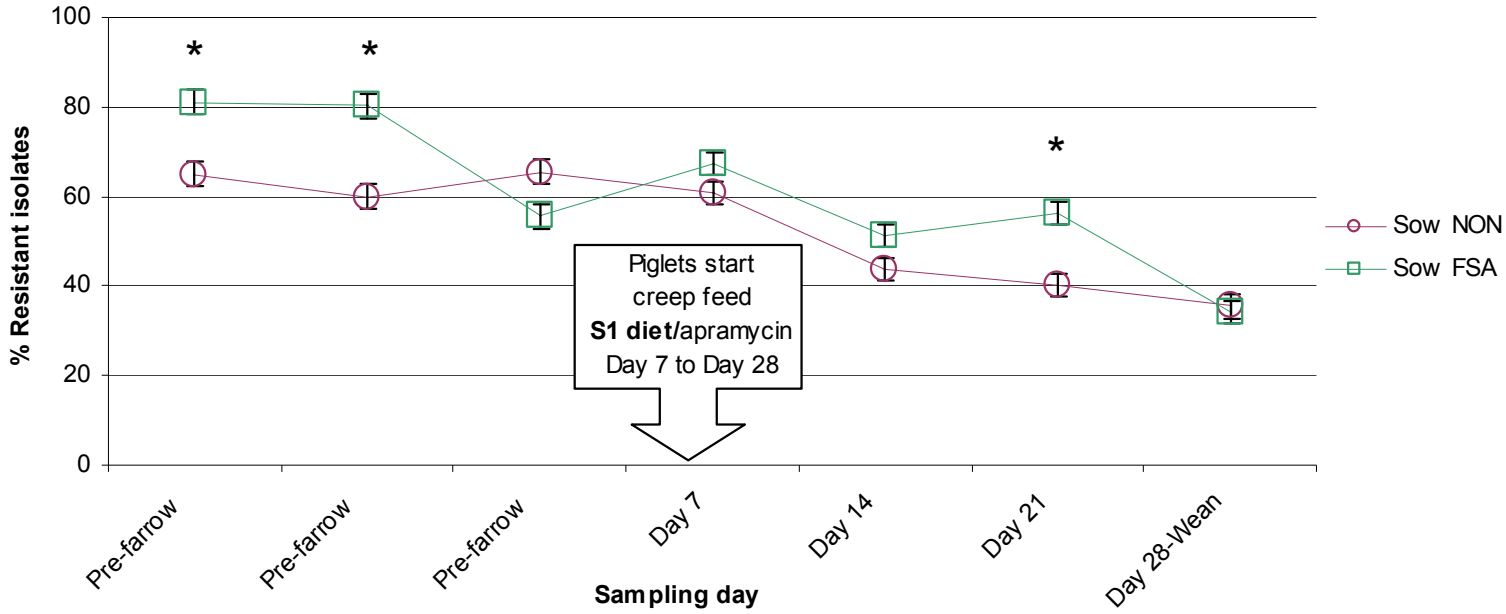


Figure 3-5. **Piglets**: total percent tetracycline resistance at 16ug/ml in *E. coli* isolates, Trials 1-3.
Statistical significance: FSA-treated piglets increased tetracycline resistance over all samples collectively, $P < 0.0001$.
Significant differences between groups on specific sampling days noted by asterisk, $*P < 0.0001$.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from control piglets, FSA=isolates from FSA-treated piglets; n=19,704.

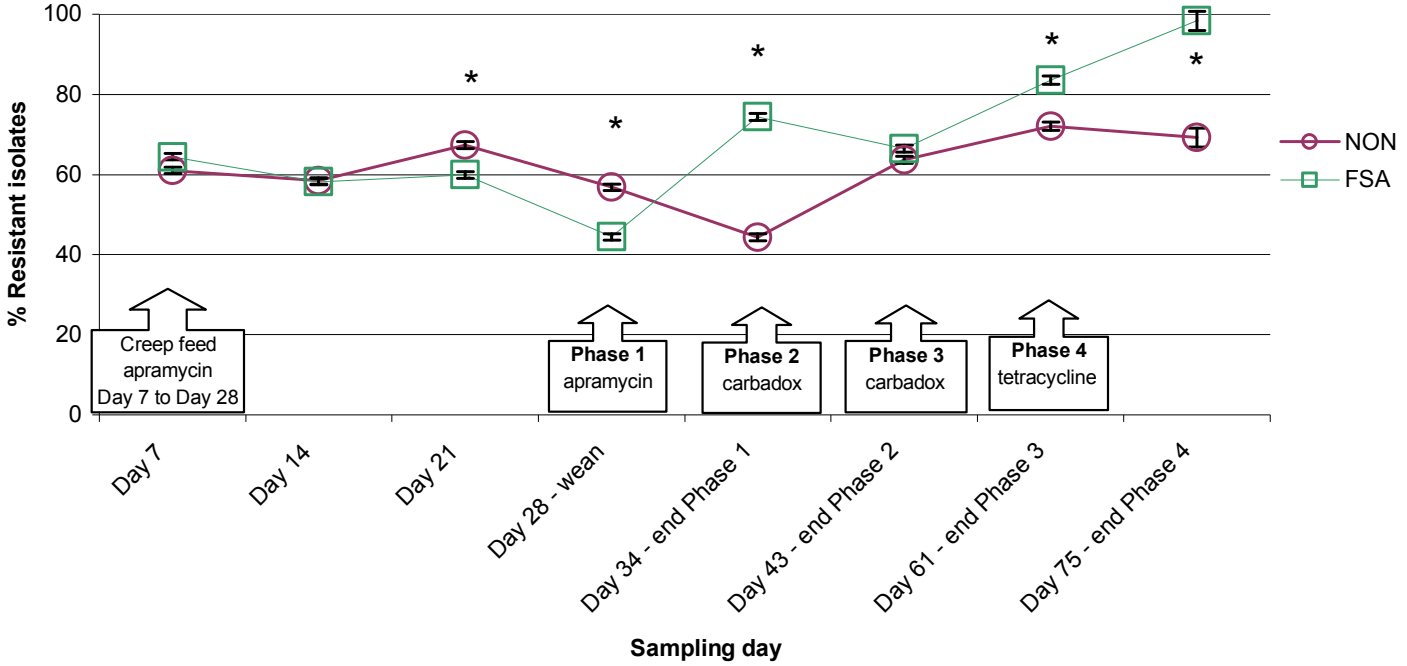


Figure 3-6. **Piglets**: total percent apramycin resistance at 32ug/ml in *E. coli* isolates, Trials 1-3.
Statistical significance: FSA-treated piglets had increased apramycin resistance over all samples collectively, $P < 0.0001$.
Significant differences between groups on specific sampling days noted by asterisk, *$P < 0.0001$.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from control piglets, FSA=isolates from FSA-treated piglets; n=19,704,.

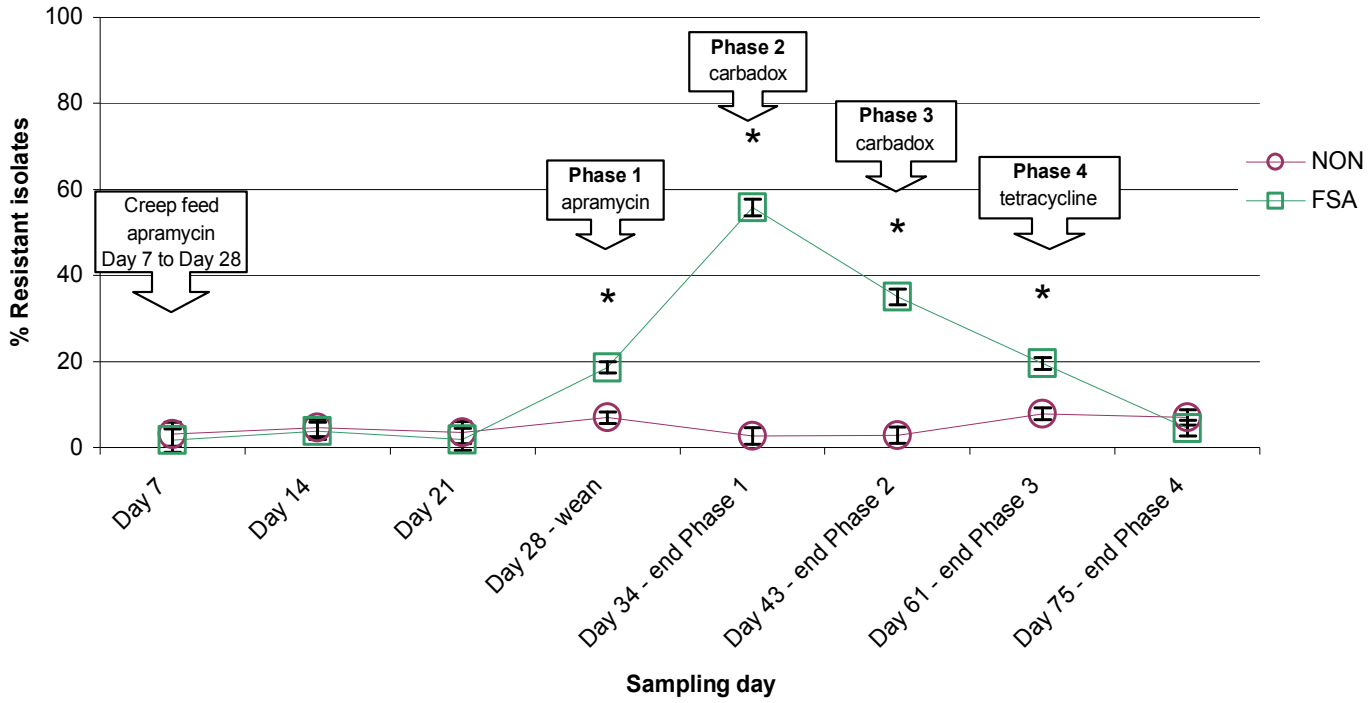
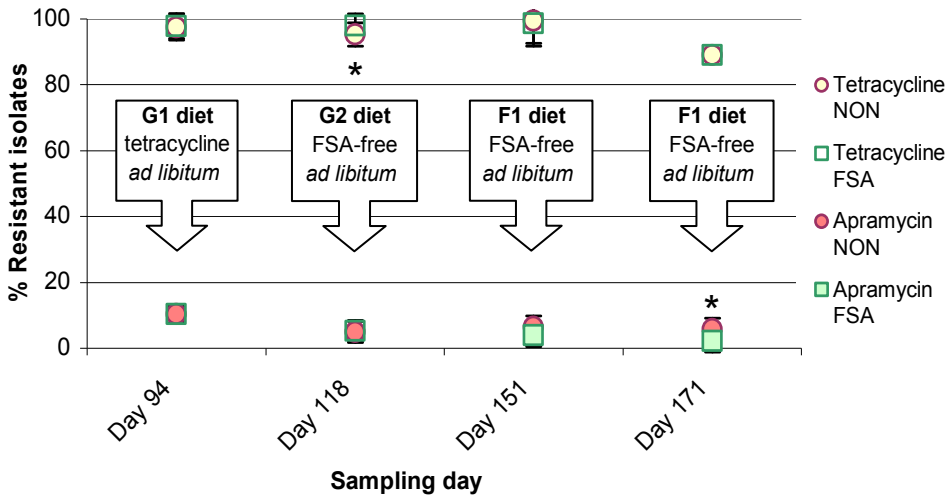


Figure 3-7. Total percent resistance to tetracycline at 16ug/ml and apramycin at 32ug/ml in *E. coli* isolates from pigs returned to the UGA Swine Center, Trials 1-3.
Statistical significance: No significant difference between groups over all samples collectively. Significant differences between groups detected on Day 118 for tetracycline, and on Day 171 for apramycin, $P < 0.05$.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from control pigs, FSA=isolates from FSA-treated pigs; n=5,531.



CHAPTER 4

EFFECT OF PORCINE-DERIVED MUCOSAL COMPETITIVE EXCLUSION ON ANTIMICROBIAL RESISTANCE IN *E. COLI* FROM GROWING PIGLETS

Introduction

One of the foremost concerns in food safety today is the increase in antimicrobial resistance among pathogens and commensal flora from humans and animals. Since the discovery of penicillin in the 1940's, antimicrobial usage in humans and livestock has increased dramatically worldwide. Use in livestock increased with the advent of confinement agriculture[1] and research in 1946 demonstrated increased animal productivity following use of subtherapeutic levels of antimicrobials in animal feeds.[2] Increased antimicrobial resistance in response to increased antimicrobial use in animal production was recognized early on and guidelines have been proposed since the 1960's in attempts to promote appropriate use in humans and livestock.[3] However, the extent to which human versus livestock use of antimicrobials contributes to antimicrobial resistance has yet to be defined.

While use of antimicrobial drugs in livestock production has made a significant impact on animal health, welfare, and productivity, interest in suitable alternatives such as pre/probiotics, organic acids, and use of cultures of normal flora or "competitive exclusion" (CE) cultures from young animals has increased significantly in the wake of the antimicrobial resistance issue.[35] Studies evaluating the efficacy of these alternatives are ongoing; however, to date, no single intervention has demonstrated results comparable to the use of subtherapeutic antimicrobials. Nurmi et al. demonstrated efficacy of inoculating chicks with unmanipulated

intestinal bacteria from adult birds in preventing infection when challenged with *Salmonella.*[36] Inhibition of colonization by bacterial pathogens is thought to be mediated by the anaerobic population of intestinal bacteria.[37] Competitive exclusion cultures are comprised in large part of anaerobic and facultative populations of bacteria, and their efficacy has been demonstrated.[38-40] Production performance of animals receiving probiotics has been variable.[35,41] Data regarding the effect of CE on animal productivity is not readily available. Additionally, there is little information regarding the effect on antimicrobial resistance in bacterial populations following use of direct-fed microbials such as probiotics or CE cultures.

In view of the significant concerns for increasing antimicrobial resistance and the need for safe, efficacious alternatives to antimicrobials the present study evaluated the effect of an undefined porcine-derived competitive exclusion (PCE) culture on both the antimicrobial susceptibility of commensal *E. coli* in growing piglets and on animal performance. The PCE culture used in this study has previously demonstrated effectiveness in decreasing *Salmonella* in challenged pigs.[38]

Materials and Methods

Animals. Sows were obtained from the University of Georgia's (UGA) Swine Center for both trials. One week prior to farrowing, sows were moved to UGA's Animal and Dairy Science Complex (ADSC) farrowing unit and randomly allotted to treatment groups. All sows were first parity PIC[a] line 42 and were artificially inseminated with PIC 280 semen. Both the gestation and lactation diets were antimicrobial-free, and sows were hand-fed for maximal feed intake (20lb plus per day) at peak lactation. Piglets were cross-fostered within 72 hours post-farrowing as needed to adjust litter size and ensure maximum survival. The farrowing and grower facilities at ADSC (Appendix B) were environmentally controlled and provided water *ad libitum* for sows

and piglets. At all times during the farrowing, lactation, and nursery phases, pigs from both groups were housed within the same rooms but on separate sides of 6 foot concrete aisles. The pull-plug pit systems below the pens were also separate and each was drained and refilled weekly. Care was exercised to handle and sample control piglets before handling those treated with PCE. At sampling, coveralls and gloves were donned upon entry to the unit and changed between groups; boot covers were used when entering pens and changed between groups. At weaning, piglets were moved to the ADSC pig growth room and sorted by weight and sex into pens of 2 to 4 piglets per pen (3 ft x 6 ft).

Experimental design. Two replicate trials (Trials 4 and 5) were conducted using different sows. Each trial was composed of 2 groups (up to 36 piglets per group). In the control group, piglets were placebo-dosed orally with sterile PRAS BHI broth (Remel, Lenexa, KS). For the treatment group, piglets were orally dosed with PCE culture (10^{10} cfu/ml). Dosing intervals and amounts as follows: 2mls each within 6 hours of birth and 18-24 hours later; 5mls each at weaning (Day 21) and 18-24 hours post-wean. Sows and piglets were sampled at regular intervals for fecal *E. coli*, and recovery of 6 *E. coli* isolates per animal was attempted at each sampling. The sampling and diet schedule is shown in Table 4-1 and feed intake was recorded throughout each trial. Piglets were raised per standard production practice guidelines established at the UGA Swine Center, including diets and duration of feeding.[b] All diets were appropriate to age and phase of growth or gestation/lactation and formulated to meet or exceed National Research Council requirements (Appendices C, D).[245] Gestation and lactation diets were antimicrobial-free, and sows were hand fed for maximal feed intake (20lb-plus per day) at peak lactation. The prestarter diet (S1) was fed in stainless steel bowls as creep feed starting on Day 7

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[a] PIC, Pig Improvement Company, 3033 Nashville Road, Franklin, KY 42135
[b] UGA Swine Center Operations Manual, ADSC Swine Center, Athens, GA, 30602

post-farrowing. At weaning (Phase 1 – Day 21) 5lb/pig was dispensed and was consumed in approximately 4 days. At Phase 2, 15lb/pig of the S2 diet was dispensed and consumed in approximately 7 days. In Phase 3, the S3 diet was fed *ad libitum* for ~10 days. Environmental samples were collected from the ADSC farrowing and grower units prior to each use as described below.

Bacteriology. The farrowing and grower facilities were sampled for *Salmonella* and *E. coli* prior to each use. Ten to 12 drag swabs pre-moistened with PBS were taken per site from crate dividers, mats, feeders, waterers, doors and aisleways, and placed in sterile Whirl-pak™ bags prior to the addition of 20mls of BHI broth (Difco, Detroit, Michigan). Fecal samples from sows were cultured for *Salmonella* and *E. coli* 7 days pre- and 21 days post-farrowing. Fecal samples were taken regularly from all piglets (Table 4-2) for culture of *E. coli* only from birth to 6-weeks of age.

Salmonella isolation was performed as previously described (Appendix E). Briefly, 1 gram of feces was diluted in 9mls PBS and 100ul transferred to 9.9mls each of tetrathionate broth and GN-Hanja broth (Difco, Detroit, Michigan). The GN-Hanja broth was incubated overnight at 37°C, and the tetrathionate broth was incubated for 48 hours at 37°C; 100ul of each was then transferred to Rapport Vassiliadis Broth R-10 (BD Microbiology Systems, Sparks, Maryland) and incubated at 37°C overnight. Each RV subculture was then plated to both xylose-lysine-tergitol 4 agar (XLT-4; BD Microbiology Systems) and brilliant green with sulfadiazine agar (BGS; Difco) and incubated overnight at 37°C. Presumptive positive colonies were inoculated onto TSI and LIA slants and incubated overnight at 37°C. Colonies presumed positive for *Salmonella* were serogrouped using *Salmonella* O-antiserum (BD Microbiology Systems) and sent to the National Veterinary Services Laboratories in Ames, IA for serotyping.

Environmental samples were processed for *Salmonella* by adding 1ml of the enriched BHI broth to 9mls PBS and transferring 100ul to 9.9mls each of tetrathionate broth and GN-Hanja broth and proceeding as above.

Escherichia coli was isolated as previously described (Appendix E). Briefly, 1 gram of feces was diluted in 9mls PBS, and a 100ul aliquot of sample was direct plated to Chromagar® ECC agar (Hardy Diagnostics, Santa Maria, California) and incubated 18-24 hours at 42°C. For piglets, one swab (used through Day 14 and for smaller piglets to Day 21) or fecal loop from each piglet was diluted in 3mls PBS then direct plated to Chromagar® ECC agar as described above. For environmental samples, 100ul of BHI broth from the drag swab was direct plated to Chromagar® ECC agar as described above, and another 100ul of the BHI broth was plated to Chromagar® ECC agar after overnight enrichment of the BHI broth at 37°C. Recovery of 6 colonies per sow or piglet was attempted, and blue-green colonies were preferentially selected (Appendix F).[247] For plates that did not contain typical blue-green colonies, non-typical colonies were selected and tested to confirm they were *E. coli* using the Vitek (bioMérieux, Inc.) biochemical test.

PCE culture. Mucosal scrapings for preparation of the PCE culture were harvested as previously described from a healthy 6-week old, *Salmonella*-free piglet.[38] The original PCE seed stock which had been stored under reduced (anaerobic) conditions at -70°C was expanded anaerobically in approximately 20 liter batches. The media was concentrated approximately 10-fold by centrifugation and refrozen in 5ml aliquots under reduced conditions at -20°C until use. The culture was thawed in warm water within one hour prior to oral dosing. Viable plate counts were performed at each PCE dosing. Briefly, 0.1ml of PCE was diluted into 0.9mls PBS and vortexed. Serial dilutions (0.1ml sample into 0.9mls PBS) were completed to 10^{-9} (Appendix N).

One hundred microlitres from the final 3 dilutions were then spread-plated to duplicate BHI agar plates (Difco), incubated at 37°C for 18-20 hours, and colonies counted manually. Final counts were averaged from duplicate plate counts and reported in $\log_{10}$ colony-forming units per ml (CFU/ml). *Escherichia coli* isolates were also isolated from the PCE culture at each dosing by plating the 10^{-2} dilution to Chromagar® ECC and proceeding as above, selecting up to 6 colonies per sample (Appendix N). Antimicrobial susceptibility testing was performed on these isolates using a 96-well custom-made panel in the Sensititre® system (Trek Diagnostic Systems, Inc., Cleveland, Ohio) per manufacturers' instructions.

Antimicrobial susceptibility. Phenotypic antimicrobial susceptibility screening of all *E. coli* isolates from sows and piglets was accomplished using a replica-plating method previously described.[248,249] The screening included 4 antimicrobials, tetracycline, ampicillin, streptomycin and apramycin. The National Committee for Clinical Laboratory Standards interpretation criteria for breakpoints was used for tetracycline (16ug/ml) and ampicillin (32ug/ml). For apramycin (32ug/ml) and streptomycin (64ug/ml), breakpoints used by the National Antimicrobial Resistance Monitoring System[c] (NARMS) were followed.[250] Colonies to be tested were transferred to a gridded EC Medium with MUG plate (Difco) and incubated overnight at 37°C. The colonies were inoculated onto a sterile felt, then replicated onto 6 Mueller Hinton II agar plates (BD Microbiology Systems) containing the antimicrobials to be tested at doubling dilutions, followed by a final antimicrobial-free plate to ensure adequate and even transfer of colonies (Appendix G). All plates were incubated overnight at 37°C. To minimize subjectivity, one person was responsible for reading all plates. Colonies were

[c] NARMS web page available at: http://www.fda.gov/cvm/index/narms/narms_pg.html

subjectively scored as 0 (no growth) and 1 (visible colony). This replica-plating method was validated in a previous study (Appendix H).

Additional antimicrobial susceptibility testing was also performed on *E. coli* from the PCE cultures described above and environmental samples, as well as *Salmonella* isolates using a 96-well custom-made panel in the Sensititre® system (Trek Diagnostic Systems, Inc., Cleveland, Ohio) per manufacturers' instructions. All isolates were stored in Mueller Hinton Broth (Difco) with 10% glycerol at –90°C for future use.

Performance. Piglets were weighed at birth and on days 7, 21 (weaning), 24, 31, and 41 (Table 4-1). Performance measurements included adjusted 21-day weights,[d] average daily gains, and feed efficiencies. Evaluation of health status including screening for parasites and detection of infectious diseases was also performed as needed.[e]

Statistical Analysis. Dichotomous antimicrobial resistance data was analyzed using regression analysis of count data for repeated measures.[251] Performance data were analyzed using GLM procedures of SAS (SAS Inst. Inc., Cary, North Carolina). Birth weight was included as a covariable in the performance models.

Results

Piglets in all trials were predominantly healthy, although minor individual health problems were encountered in piglets from both treatment groups including scours, sternal abscesses, and sneezing. During Trial 4, one healthy barrow from the control group was returned to the Swine Center at weaning due to insufficient pen space in the grower room. One piglet from Trial 5 died in the farrowing room, trapped under the sow's feeder.

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[d] National Swine Improvement Foundation web page available at: http://mark.asci.ncsu.edu/nsif/guidel/guidelines.htm

[e] Athens Diagnostic Laboratory, University of Georgia, Athens, GA 30602

Significant differences in feed efficiency were identified in Phases 1 and 2 for both trials (Figure 4-1). Piglets treated with the PCE culture demonstrated improved feed efficiencies when compared to control piglets ($P < 0.005$). Although not statistically significant, improved feed efficiencies were also noted in PCE-treated piglets during Phase 3 of Trial 4. Average daily gains (Appendix O) and adjusted 21-day weights (data not shown) also tended to be higher in PCE-treated piglets than controls; however the difference was not statistically significant.

For both trials, *E. coli* were recovered from 99.0% (499/504) of sow samples, 98.7% (6276/6360) of piglet samples, and 50.0% (22/44) of environmental samples. *Salmonella* were isolated only from sows during Trial 4 with 7.1% (3/42) of samples positive from 3 of 6 sows. Environmental samples from both trials and sow samples from Trial 5 were negative for *Salmonella*.

All *Salmonella* isolates were identified as *S.* Thompson (group C1) and were pansensitive. Two isolates were recovered from sows in the control group, and 1 isolate was from a sow in the treatment group. Isolates were recovered on days 0 (2 isolates) and 3 (1 isolate). *Salmonella* was not recovered from any of these sows in the remaining samplings. Neither sows nor piglets exhibited clinical signs of salmonellosis during any of the trials.

Twenty to 24 environmental samples were taken from the farrowing and grower facilities prior to stocking in each trial. Antimicrobial susceptibility profiles from *E. coli* (Figure 4-2) exhibited resistance primarily to tetracycline. Only 4 isolates were resistant to more than one drug. Of these multiply-resistant *E. coli* isolates, one recovered during Trial 4 from a farrowing room mat was resistant to amoxicillin-clavulonic acid, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, naladixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim sulfamethoxazole. This isolate

possessed the antimicrobial resistance gene CMY-2[256] conferring resistance to extended spectrum cephalosporins, but was negative via polymerase chain reaction for virulence genes including shiga toxin-1, shiga toxin-2, heat-labile enterotoxin, heat-stable enterotoxin, and the protein intimin encoded by *eaeA*, an attaching and effacing protein.[257] Presence of this isolate in the environment did not appear to affect antimicrobial susceptibilities of animal *E. coli* populations during either trial.

Antimicrobial susceptibility from *E. coli* isolated from the PCE culture (n=107) is presented in Figure 4-3. The majority of isolates (84%) were resistant to tetracycline or streptomycin and tetracycline. Seven and a half percent of isolates were pansensitive. Few isolates (n=8) exhibited resistance to ampicillin, kanamycin, cephalothin, and sulfamethoxazole. The viable plate counts for the PCE culture were consistent throughout the study and ranged from 9.75-9.83 $\log_{10}$ CFU/ml (Figure 4-4). Concentration of PCE culture delivered at farrowing (2mls/piglet, Table 1) ranged from 9.9-10.3 $\log_{10}$ CFU per dose (0.8-1.8 x 10^{10} CFU) and at weaning (5mls/piglet) ranged from 10.3-10.7 $\log_{10}$ CFU per dose (2.0-4.5 x 10^{10} CFU).

In total 6,775 *E. coli* isolates from 12 sows and 132 piglets were screened (Trial 4: n=3461, Trial 5: n=3014). Resistance to tetracycline, streptomycin, ampicillin, and apramycin in total *E. coli* from sows and piglets is presented in Table 4-2. Differences between sow groups were not significant over all samples for any of the antimicrobials tested; however, sows of PCE-treated piglets had significantly more *E. coli* resistance to tetracycline on Days -2, 14, and 21 ($P < 0.05$, Figure 4-5). A significantly higher proportion of *E. coli* from PCE-treated piglets demonstrated resistance to tetracycline ($P < 0.0001$, Figure 4-6), and streptomycin ($P < 0.0001$, Figure 4-7) when compared to controls. Resistance to streptomycin resistance in *E. coli* from piglets treated with PCE culture was variable, initially increasing in PCE-treated piglets

subsequent to the first 2 doses (Days 0-1, $P < 0.0001$) and then returning to baseline levels by Day 21 (weaning). Significant differences for streptomycin were observed again after the second 2 doses at weaning (Days 21-22, $P < 0.0001$), but had not returned to baseline levels by the end of the study (Day 41). Resistance was low for ampicillin (3.3-3.7% in piglets) and rare for apramycin (0-0.2% in piglets). Significant differences were not detected between groups for these antimicrobials.

Discussion

Use of subtherapeutic levels of antimicrobials in animal feeds is thought to aid in disease prevention and promote growth.[4-8] However, subtherapeutic use of antimicrobials can produce resistance and also applies selective pressure which assists in the maintenance of resistant populations of bacteria.[9-12] Concerns regarding increased antimicrobial resistance among foodborne pathogens have led to the invocation of the precautionary principle in Europe resulting in the removal of subtherapeutic antimicrobial use in animal feeds.[13-15] These changes have renewed interested in the development of alternatives to antimicrobials such as prebiotics, organic acids, and direct-fed microbials including probiotics and CE cultures. Direct-fed microbials have been shown to decrease target pathogens by competing for attachment sites and nutrients.[36,40] Comprehensive *in vivo* evaluation of direct-fed microbials (DFM) such as probiotics and CE cultures should include assurance that the DFM is free from potential pathogens, that it demonstrates efficacy in decreasing target pathogens, has positive effects on animal performance, and has minimal impact on the antimicrobial susceptibility of commensal flora.[258] While characterization of nonpathogenicity, survival through regions of the gut, host specificity, and adherence have been reported for several probiotic organisms,[202,208,259,260] there is

little information regarding the impact of DFMs on commensal flora, including antimicrobial susceptibility profiles.[261]

Competitive exclusion cultures have been most effective in decreasing *Salmonella*[39,40] and the PCE culture used in the present study has previously been shown to effectively exclude *Salmonella* in pigs.[38] However, there remains a paucity of information regarding antimicrobial susceptibility profiles of CE organisms and commensal flora.

As a CE culture is originally propagated from a donor animal there is a need to determine how the CE culture impacts the commensal flora and its antimicrobial susceptibility. It is important to note that CE cultures for use in pigs are typically generated from 6-week or older animals with the intent of propagating established commensal flora that can be used to speed the maturity of commensal flora in younger, more naïve animals, or to assist in re-establishing populations in animals with disrupted flora due to stress or illness. Since resistance to tetracycline, streptomycin, and sulfonamides is common among gram-negative commensal bacteria isolated from pigs[32-34] it would not be unexpected to observe resistance to these antimicrobials in a CE culture.

In the present study, *E. coli* from the environment also exhibited resistance to tetracycline, streptomycin, kanamycin, ampicillin and other antimicrobials (Figure 4-2) suggesting that these resistance patterns are common among swine herds regardless of geography. Additionally, resistance to tetracycline, streptomycin, sulfamethoxazole, and other antimicrobials has been demonstrated on a farm in which antimicrobials had not been used for over 10 years.[173] Therefore, while an 'ideal' CE culture might be described as one harboring no antimicrobial resistant organisms while maintaining efficacy, it is unlikely that such a culture can be obtained from swine and still be representative of mature intestinal flora.

Current dogma for obtaining efficacious CE cultures entails harvesting mucosal scrapings from a young healthy animal of the species to be inoculated, known to be free of pathogens and viruses; continuous flow or other propagation via culture methods under reduced or anaerobic conditions; and per oral dosing early in life and/or at times of stress such as weaning, transportation, or during clinical illness. Using these criteria, the PCE culture used in the present study was harvested from a healthy 6-week old piglet under reduced conditions. The anaerobic conditions are maintained to ensure adequate viability of the anaerobic portion of the culture.[37] However, the analysis of antimicrobial resistance in this study was only performed on *E. coli* as this organism has been widely used in antimicrobial resistance monitoring[32,68-70] and is a predominant gram-negative facultative bacteria in commensal flora.[52,53] Viability counts performed for this study were under aerobic conditions and most likely does not accurately reflect the anaerobic population. The dosing regime was selected for maximal effect at birth and weaning as previously demonstrated by Fedorka-Cray et al.[38] The potential for improved feed efficiency in PCE-treated piglets was not unexpected in view of the beneficial effects found with probiotics.[262,263] However, this is the first controlled study documenting these effects in swine. It is interesting to note that while some reports of improved performance with use of subtherapeutic antimicrobials have been observed in field conditions,[197] the present study identified significant differences in a controlled, optimal environment. These results suggest the possibility for greater improvements in performance for pigs under field conditions.

The observed increase in tetracycline and streptomycin resistance in *E. coli* from PCE-treated pigs is difficult to fully interpret as samples were not taken on Day 0 (farrowing). However, several noteworthy observations can be made. The most obvious is that the CE culture itself replaced the commensal flora of PCE-treated piglets resulting in establishment of an *E. coli*

population that was more resistant to tetracycline and streptomycin. While the culture most likely does affect the composition of the flora, this is best observed for streptomycin and not for tetracycline. Analysis of Figure 4-7 indicates that by 72h post-administration of the PCE culture, the percent of isolates resistant to streptomycin increased. Interestingly, resistance returned to baseline levels by Day 21. As the study ended at Day 41, no conclusion can be drawn regarding the increase in streptomycin resistance following the 3rd and 4th PCE doses at weaning, but is seems likely that levels would return to baseline as well.

Since there was no significant difference in resistance among sows overall, vertical transfer of resistant isolates to piglets is difficult to assess. However, increases in tetracycline resistance noted on Days -2, 14, and 21 for sows of the PCE-treated piglets suggest the possibility that the PCE-piglets were exposed to greater numbers of tetracycline-resistant *E. coli* than controls. As there was no observed increase in tetracycline resistance in the PCE-treated piglets within 72h post-administration of the PCE-culture, it is less likely that the PCE culture itself was soley responsible for the observed increase in tetracycline resistance. The combined influence of *E. coli* from the sows and the environment (Figure 4-2), in addition to the PCE culture, all of which harbored tetracycline resistance likely contributed to the increased tetracycline resistance observed in the PCE-treated pigs. This also suggests that tetracycline resistant *E. coli* may have a preferential colonization advantage and warrants further study. However, of most importance is the observation that use of subtherapeutic antimicrobials in animal production results in higher numbers of tetracycline resistant *E. coli* (Figures 3-5 and 3-7) than that observed following use of the PCE culture. Futher studies are warranted with other CE cultures to determine if lower levels of resistant *E. coli* can be established and maintained, especially in light of the observed increase in feed efficiency in PCE-treated piglets (Figure 4-1).

Current literature suggests that a successful move from subtherapeutic antimicrobial use to suitable alternatives will require a combination of precise nutrition, proper management and sanitation, careful record keeping, and appropriately applied supplements at key points in growth.[35,41,42] A recent epidemiologic report proposes that subtherapeutic antimicrobials be limited to use during the nursery phase in swine production where they have optimal effect.[253] Reducing subtherapeutic antimicrobial use to this phase in swine production alone would likely impact the amount of antimicrobial drugs used.[264] Appropriate application of subtherapeutic antimicrobials combined with strategic supplementation with alternatives such as probiotics and CE cultures may allow for significant decreases in antimicrobial use in swine production without sacrificing animal health, welfare and productivity.

Implications: Results from this study suggest that CE cultures may improve animal performance and reduce incidence of certain diseases. However, alteration of the commensal flora can be expected, which may also affect the populations of *E. coli* resistant to some antimicrobials. Appropriate application of subtherapeutic antimicrobials combined with strategic supplementation with alternatives such as probiotics and CE cultures may allow for significant decreases in antimicrobial use in swine production without sacrificing animal health, welfare and productivity.

Table 4-1. Sampling schedule for piglets.

Day	PCE	Diet: Sows	Diet: Piglets	Animals sampled	Weights	Other procedures
-7 to 2	Dose **2ml** 2-6hr and 18-24hrs post-farrow	Gestation diet	--	Sows prior to farrowing	Birth weight	Process within 24 hrs of birth: ear notch for identification, dock tails, clip milk teeth, 1ml iron[a] IM
3		Lactation diet	Creep feed **S1** diet	Piglets and sows	--	--
7		Lactation diet	Creep feed **S1** diet	Piglets and sows	Weight	Vaccinate[b] piglets IM, castrate males, and start creep feed
14		Lactation diet	Creep feed **S1** diet	Piglets and sows		Piglets ~14 days of age; vaccinate[c] sows IM
21 Wean	Dose **5ml**	--	**S1** diet 5lbs/pig	Piglets and last sample for sows	Weight at weaning	Wean piglets and re-vaccinate; sows return to farm
22	Dose **5ml**	--	**S1** diet	Piglets	--	--
24 end **Phase 1**		--	**S1** diet	Piglets	Weight at end of S1 diet	--
31 end **Phase 2**		--	**S2** diet 15lbs/pig	Piglets	Weight at end of S2 diet	--
41 end **Phase 3**		--	**S3** diet *ad libitum*	Piglets	Weight at end of S3 diet	--

[a] Ferrodex 100 Iron hydrogenated dextran 100mg/ml; AgriLabs, St. Joseph, MO 64503
[b] ProSystem® B*P*M*E *Bordetella bronchiseptica*, *Pasteurella multocida*, *Mycoplasma hyopneumoniae* bacterin, *Erysipelothrix rhusiopathiae* bacterin toxoid (U.S. Patent 5,338,543); Intervet, Inc., Millsboro, DE 19966
[c] Breed Sow 7 Parvovirus vaccine – killed**,** *Erysipelothrix rhusiopathiae; Leptospira canicola, grippotyphosa, hardjo, icterohemorrhagica, pomona* bacterin toxoid, AgriLabs, St. Joseph, MO 64503

Table 4-2. Total percent resistance in *E. coli* from sows and piglets by treatment group. Significant differences noted by asterisk, *P < 0.0001.

Antimicrobial	Percent resistance			
	Isolates from sows of control piglets n=250	Isolates from sows of PCE-treated piglets n=249	Isolates from control piglets n=2764	Isolates from PCE-treated piglets n=3512
Tetracycline 16ug/ml	63.7	70.9	77.8	* 93.0
Streptomycin 64ug/ml	25.4	36.9	20.5	* 39.6
Ampicillin 32ug/ml	7.3	18.3	3.3	3.7
Apramycin 32ug/ml	0.5	0.0	0	0.2

Figure 4-1. Feed efficiencies (FE) for piglets in Trials 4-5.
Statistical significance: Piglets treated with the porcine competitive exclusion culture (PCE) had significantly improved FE in Phases 1 and 2 (noted by asterisk), *P < 0.005.
Error bars represent 1 standard error from statistical model.
Groups: NON=control piglets, PCE=PCE-treated piglets; n=132.

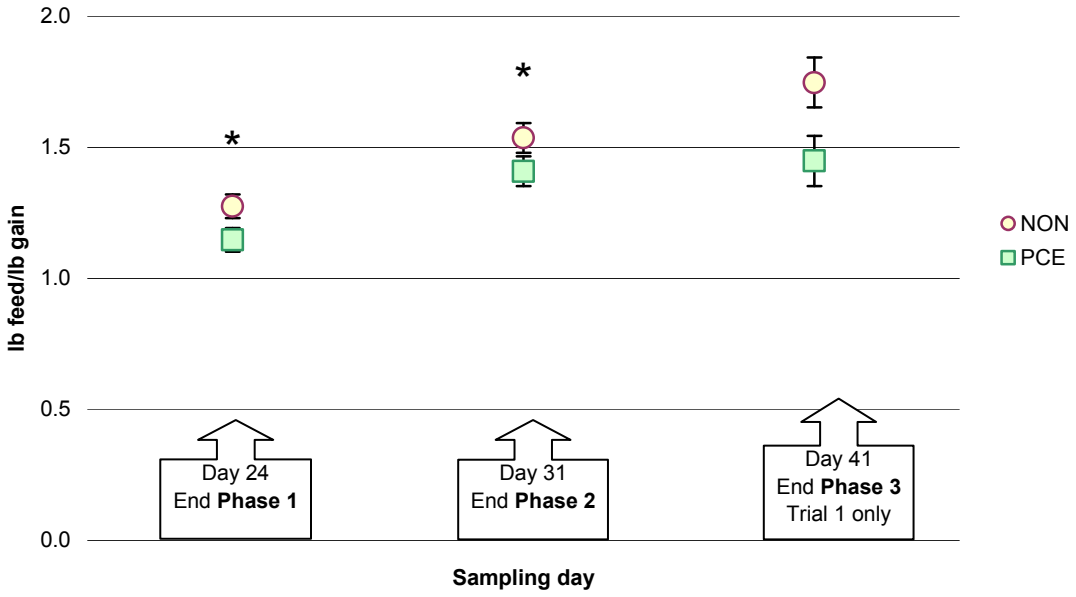
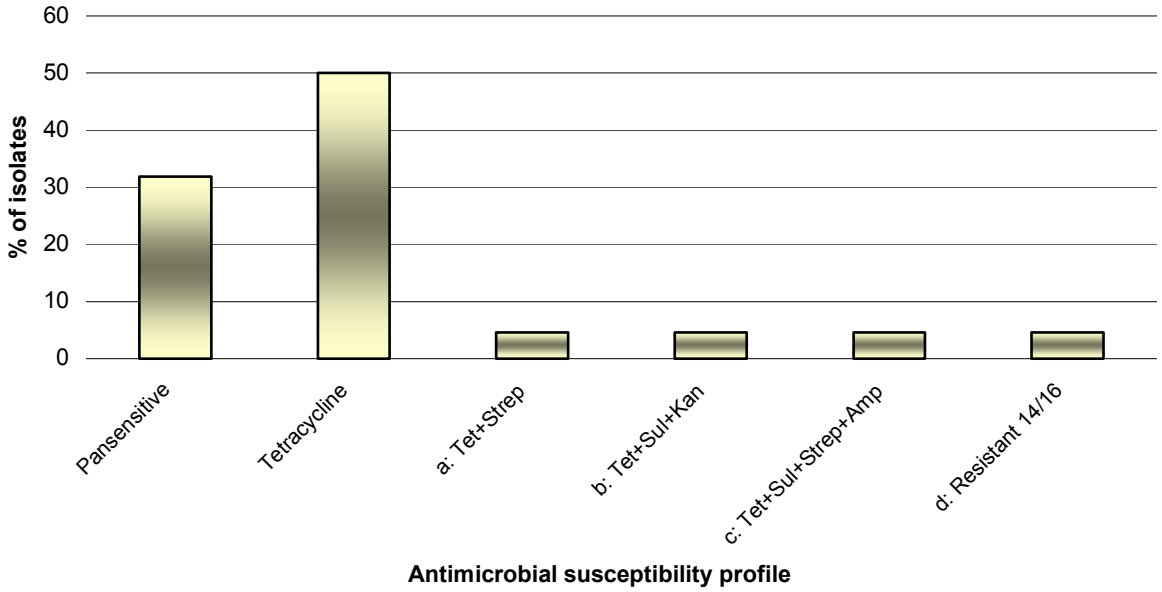


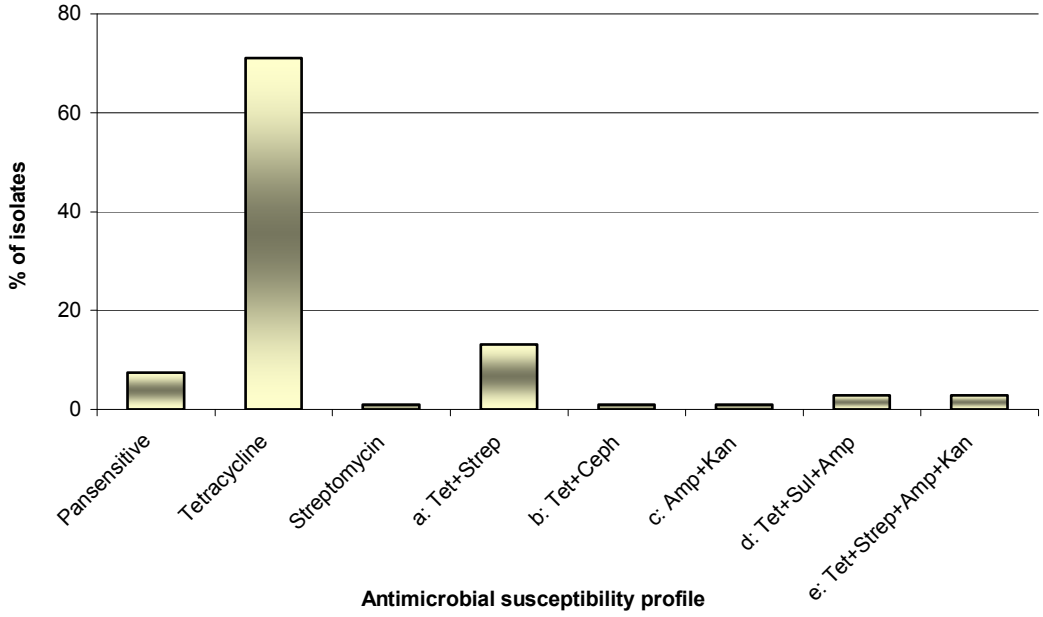
Figure 4-2. Antimicrobial susceptibility profiles of *E. coli* isolates from environmental samples, n=22.[♦]
Note: Scale to 60% only.



a: tetracycline and streptomycin
b: tetracycline, sulfamethoxazole, and kanamycin
c: tetracycline, sulfamethoxazole, streptomycin, and ampicillin
d: amoxicillin-clavulonic acid, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamyicn, naladixic acid, streptomycin, sulfamethoxazole, tetracycline, trimethoprim sulfamethoxazole

[♦] 'Resistant 14/16' represents a single isolate recovered during Trial 4 resistant to 14 of 16 antimicrobials tested

Figure 4-3. Antimicrobial susceptibility profiles of *E. coli* isolates from the porcine mucosal competitive exclusion culture (PCE); n=107.
Note: Scale to 80% only.



a: tetracycline and streptomycin
b: tetracycline and cephalothin
c: ampicillin and kanamycin
d: tetracycline, sulfamethoxazole and ampicillin
e: tetracycline and streptomycin, ampicillin, and kanamyicn

Figure 4-4. Concentration expressed in $\log_{10}$ colony forming units (CFU) per ml for the porcine mucosal competitive exclusion culture (PCE).
Error bars represent 1 standard deviation from the mean.

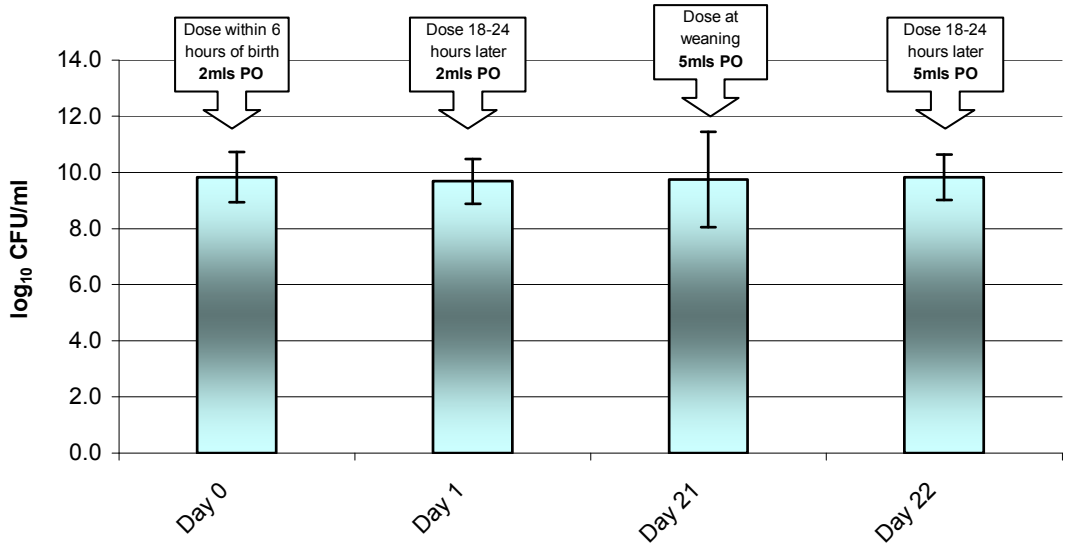


Figure 4-5. **Sows**: total percent tetracycline resistance at 16ug/ml in *E. coli* isolates, Trials 4-5.
Statistical significance: No significant difference between groups over all samples collectively. Sows of PCE-treated piglets had significantly increased tetracycline resistance at Days -2, 14, and 21 (noted by asterisk), *P < 0.05.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from sows of control piglets, PCE=isolates from sows of PCE-treated piglets; n=499.

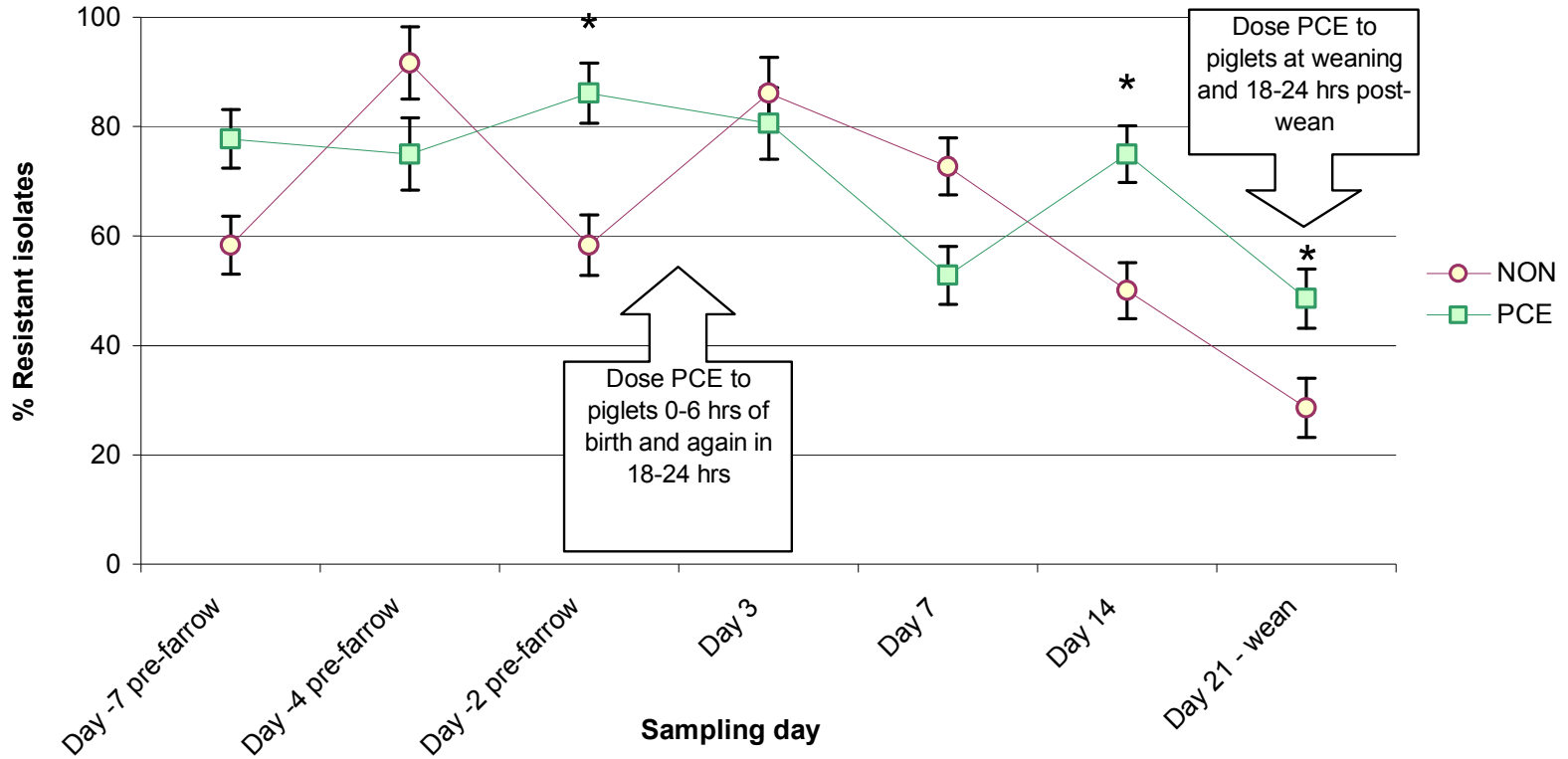


Figure 4-6. **Piglets**: total percent tetracycline resistance at 16ug/ml in *E. coli* isolates, Trials 4-5.
Statistical significance: Tetracycline resistance was increased in PCE-treated piglets over all samples collectively, P<0.0001.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from control piglets, PCE=isolates from PCE-treated piglets; n=6,276.

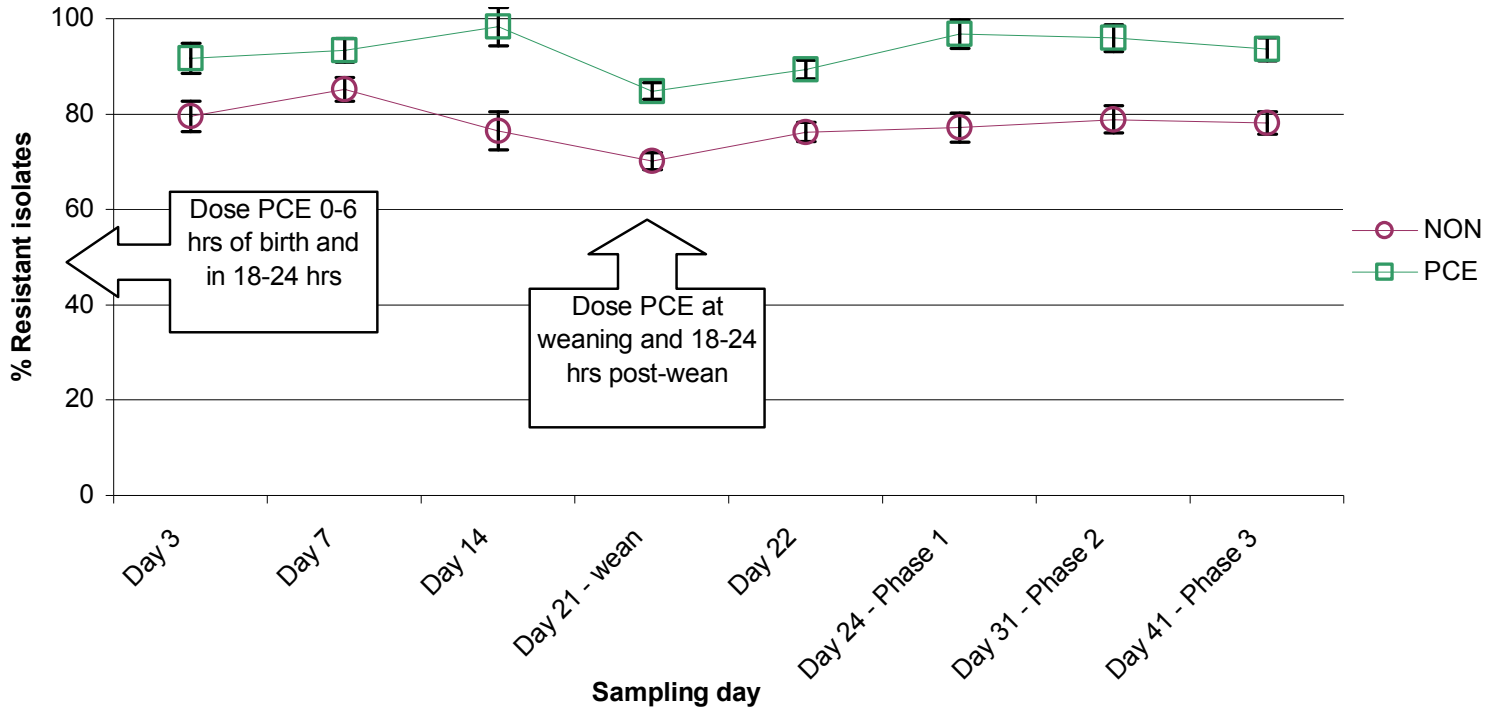
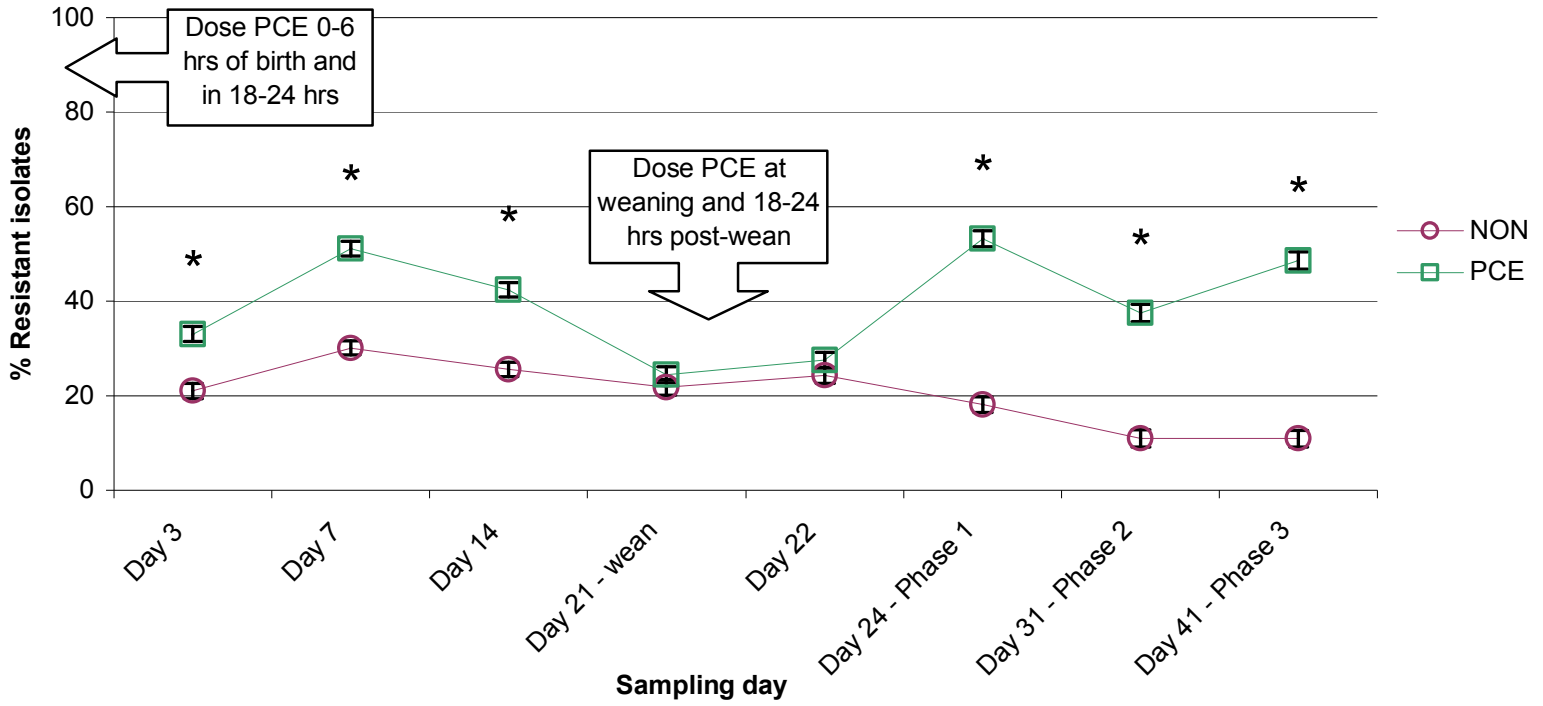


Figure 4-7. **Piglets**: total percent streptomycin resistance at 64ug/ml in *E. coli* isolates, Trials 4-5.
Statistical significance: Streptomycin resistance was increased in PCE-treated piglets over all samples collectively, $P < 0.0001$.
Significant differences between groups on specific sampling days noted by asterisk, $*P < 0.0001$.
Error bars represent 2 standard errors derived from the statistical model.
Groups: NON=isolates from control piglets, PCE=isolates from PCE-treated piglets; n=6,276.



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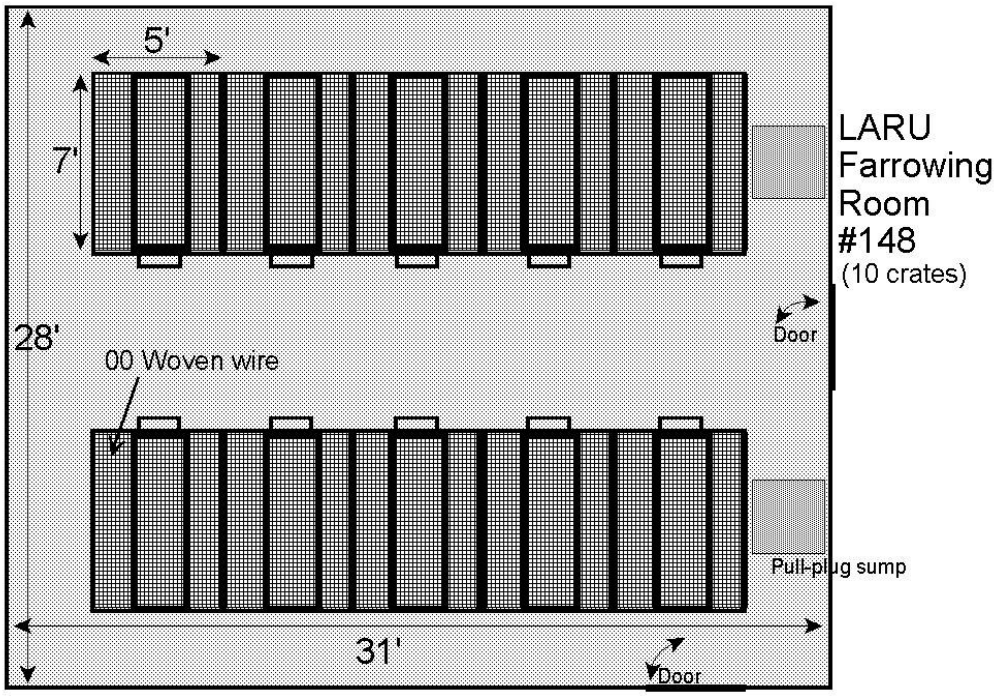
APPENDICES

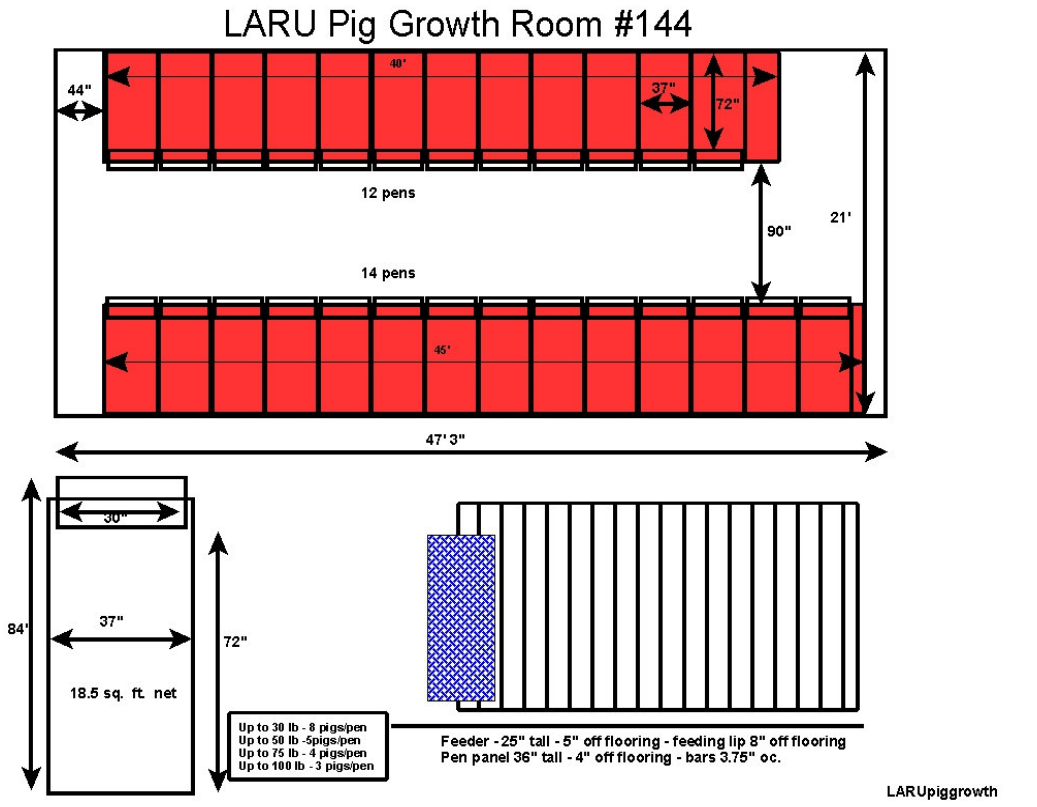
Appendix A. Sow genetics for Trials 1-3. Non=sows of control piglets, FSA=sows of FSA-treated piglets.

Trial	Group	ID	Genetics	Parity	# Piglets
1	Non	608	H-L x LW	1	10
	Non	588	H-L x LW	2	8
	Non	595	H-L x LW	2	3
	Non	570	H-L x LW	4	10
	FSA	580	H-L x LW	3	7
	FSA	594	H-L x LW	2	11
	FSA	593	H-L x LW	2	7
	FSA	581	Landrace x Welsh	3	6
2	Non	589	H-L x LW	3	10
	Non	607	H-L x LW	1	7
	Non	619	H-L x LW	1	10
	Non	577	Landrace x Hampshire	4	9
	FSA	606	H-L x LW	2	12
	FSA	618	H-L x LW	1	11
	FSA	569	Landrace x Hampshire	4	10
3	Non	741	Y-H-LW x DRU	2	12
	Non	653	Y-H-LW x DRU	5	11
	Non	676	Y-H-LW x DRU	5	10
	Non	672	Y-H-LW x DRU	5	10
	FSA	678	Y-H-LW x DRU	5	9
	FSA	677	Y-H-LW x DRU	5	11
	FSA	695	Y-H-LW x DRU	4	13
	FSA	737	Y-H-LW x DRU	2	5

H-L x LW: Hampshire-Large White by Large White
Y-H-LW x DRU: Yorshire-Hampshire-Large White by DRU

Appendix B. Farrowing and grower facilities at the University of Georgia's Animal and Dairy Science Center (ADSC).





Appendix C. Formulations for gestating and lactating sow diets.

	Percent (%) minimum in diet	
	Gestation	Lactation
Amino acid content: Lysine	0.78	1.04
Methionine and Cystine	0.48	0.58
Tryptophan	0.15	0.20
Threonine	0.51	0.64
Crude Protein	13.00	16.54
Crude Fat	6.77	8.96
Crude Fiber	6.74	6.77
Calcium	0.90	0.90
Calcium (% maximum in diet)	0.90	0.90
Phosphorus	0.75	0.80
Metabolizable Energy (kcal/lb)	1481.0	1525.0

Appendix D. Formulations for piglet diets – ***Note***: all diets are ground except for S1 and S2 which were pelleted; G2 and F1 diets are the same for all pigs and were only fed at the UGA Swine Center.

	Percent (%) minimum in diet					
	S1	S2	S3	G1	G2	F1
Amino acid content: Lysine	1.50	1.35	1.25	1.00	0.80	0.75
Methionine and Cystine	0.90	0.81	0.75	0.62	0.52	0.51
Tryptophan	0.28	0.25	0.24	0.20	0.16	0.15
Threonine	1.07	0.88	0.81	0.68	0.58	0.56
Crude Protein	21.68	20.59	19.86	16.34	13.57	12.98
Crude Fat	5.10	5.68	5.67	7.58	7.63	6.25
Crude Fiber	1.50	1.98	2.36	2.25	2.18	2.20
Calcium	0.90	0.90	0.75	0.75	0.70	0.65
Calcium (% maximum in diet)	0.90	0.90	0.75	0.75	0.70	0.65
Phosphorus	0.80	0.80	0.70	0.65	0.60	0.50
Metabolizable Energy (kcal/lb)	1500.00	1500.00	1500.00	1550.00	1550.00	1525.00

Appendix E. Detailed bacterial culture.

	Salmonella	***E. coli***
Day 1	▪ 1 gm (swab) of feces into 9 mls tetrathionate broth – vortex ▪ Incubate 48 h at 37°C ▪ 1 gm (swab) of feces into 9 mls GN Hanja broth – vortex ▪ Incubate 18-24 h at 37°C	▪ 1 rectal swab or fecal oop from piglet into 3 mls PBS (1:4 dilution) [sows~1gm feces on swab to 9 mls PBS (1:10)] – vortex ▪ Inoculate 100 ul (swab) to Chromagar®, streak for isolation ▪ Incubate 24 h at 42°C
Day 2	▪ Continue 48 h tetrathionate incubation at 37°C ▪ Transfer ~100μl via swab of the GN enrichment to Rappaport R-10 media (discard GN) ▪ Incubate 18-24 h at 37°C	▪ Grid 12 presumptive colonies per sample onto EC Medium with MUG agar plates ▪ Incubate 18-24 h at 37°C
Day 3	▪ Transfer ~100μl via swab of the tetrathionate enrichment to Rappaport R-10 media (discard tetrathionate) ▪ Incubate 18-24 h at 37°C ▪ Streak GN/Rappaport 48h enrichment onto Brilliant Green agar w/ sulfadiazine (BGS) and xylose-lysine-tergitol 4 agar (XLT-4) ▪ Incubate 18-24 h at 37°C	▪ Replicate gridded MUG plates onto Mueller Hinton (MH) agar with selected antimicrobials for antimicrobial susceptibility screening: 7 MH plates were used for each replicate: ▪ Tetracycline at 4ug and 16ug ▪ Apramycin at 8ug and 32ug ▪ Gentamicin at 4ug and 16ug ▪ Control plate (MH with no additive) ▪ Incubate 18-24 h at 37°C
Day 4	▪ Streak tetrathionate/ Rappaport 48h enrichment onto BGS and XLT-4 agar ▪ Incubate 18-24 h at 37°C ▪ Inoculate presumptive colonies from GN/Rappaport BGS and XLT-4 plates on triple sugar iron (TSI) and lysine iron agar (LIA) slants ▪ Incubate 18-24 h at 37°C	▪ Read MH plates ▪ Transfer from gridded MUG plate into 96-well plates with MH broth plus 10% glycerol ▪ Store at -70°C
Day 5	▪ Inoculate presumptive colonies from tetrathionate/Rappaport BGS and XLT-4 plates on TSI and LIA slants ▪ Incubate 18-24 h at 37°C ▪ Read GN/Rappaport TSI and LIA slants	
Day 6	▪ Read tetrathionate/Rappaport TSI and LIA slants ▪ Group presumptive colonies off XLT-4 plates – streak to nutrient agar - Incubate 18-24 h at 37°C ▪ Send NA slant for serotyping at NVSL	**Note**: each batch of MH plates was checked with appropriate quality control strains

Appendix F. Typical blue-green *E. coli* colony on Chromagar® ECC agar.

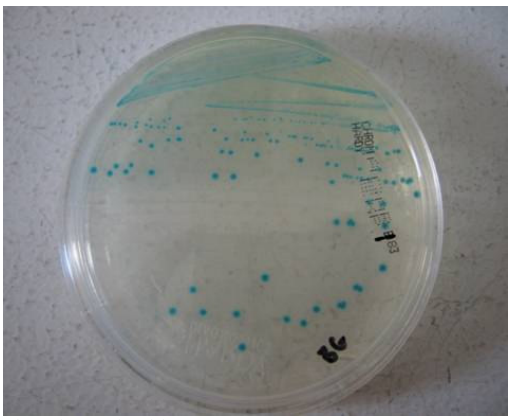
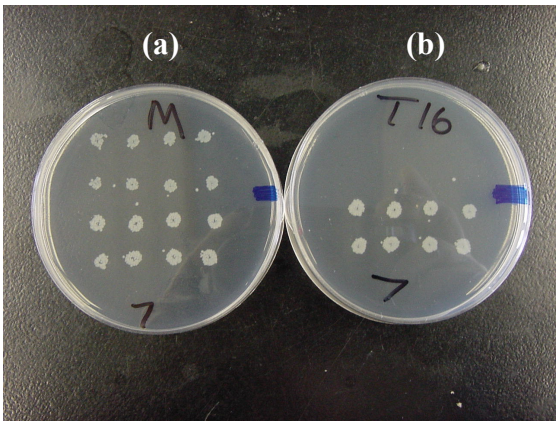


Photo by J. Plumblee

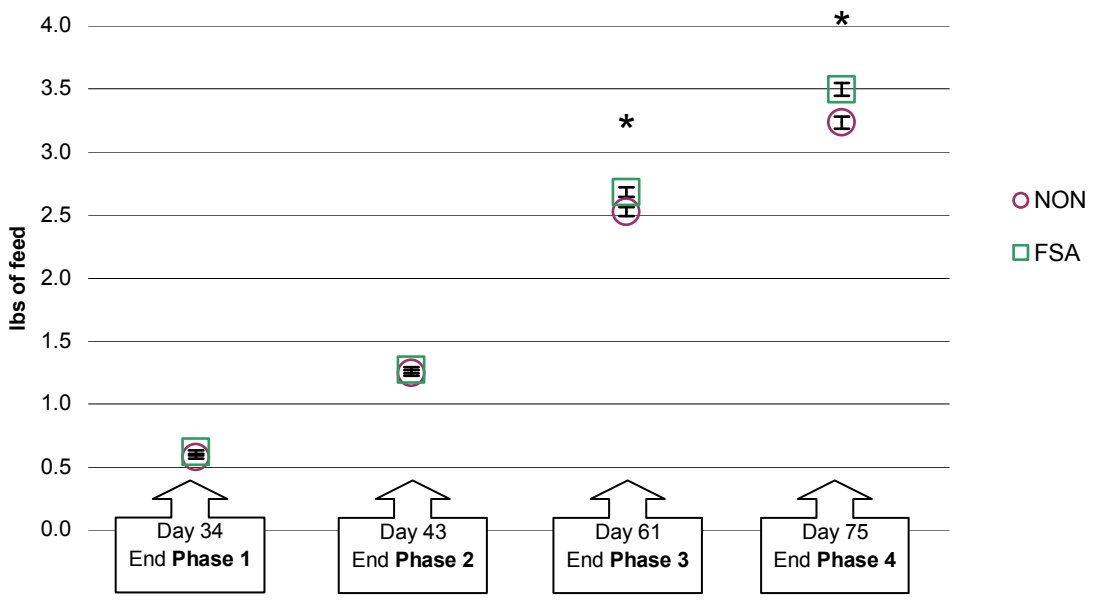
Appendix G. *E. coli* isolates replicated to (a) Mueller Hinton II (MH) agar, and (b) MH agar with tetracycline at 16ug/ml.



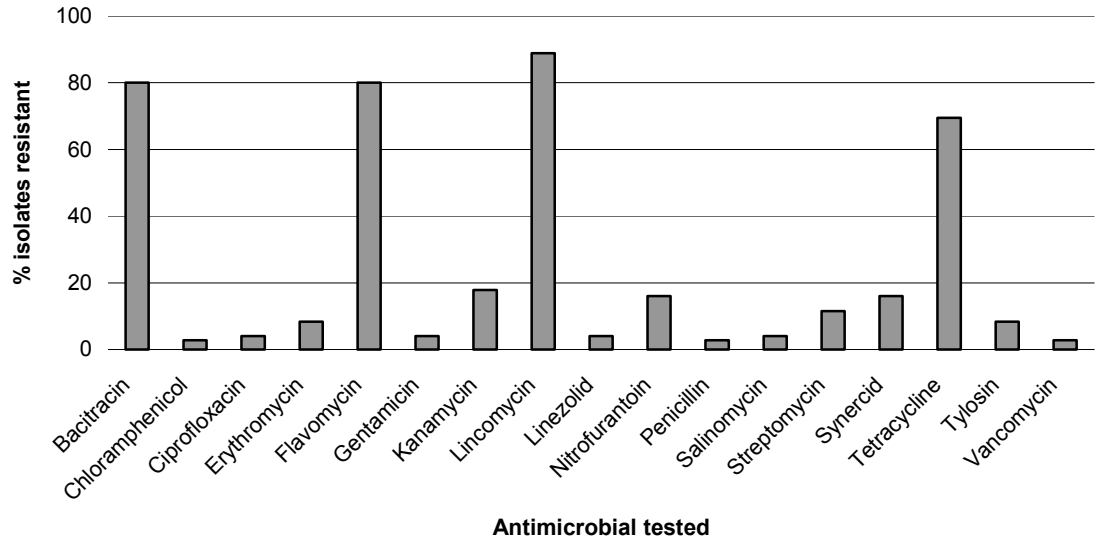
Appendix H. Total percent sensitivity and specificity of replica-plating results compared to results from Sensititre® susceptibility testing: apramycin, n=165; tetracycline and gentamicin, n=580.

Antimicrobial	*% Sensitivity*	*% Specificity*
Apramycin	83.33	94.34
Gentamicin	98.33	90.05
Tetracycline	94.08	89.23

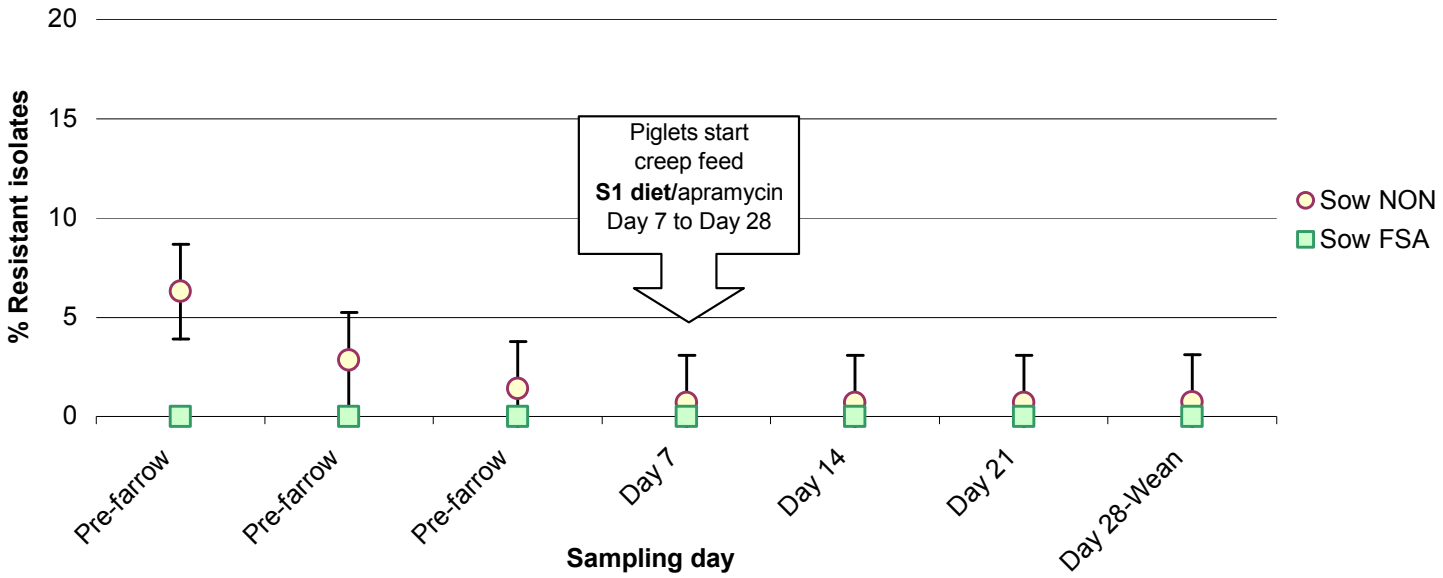
Appendix I. Daily feed intakes (DFI) for piglets in Trials 1-3.
Statistical significance: FSA-treated piglets had significantly higher DFIs in Phases 3 and 4 (noted by asterisk) *P < 0.0001.
Error bars represent 1 standard error derived from the statistical model.
Groups: NON=control piglets, FSA=FSA-treated piglets; n=204.



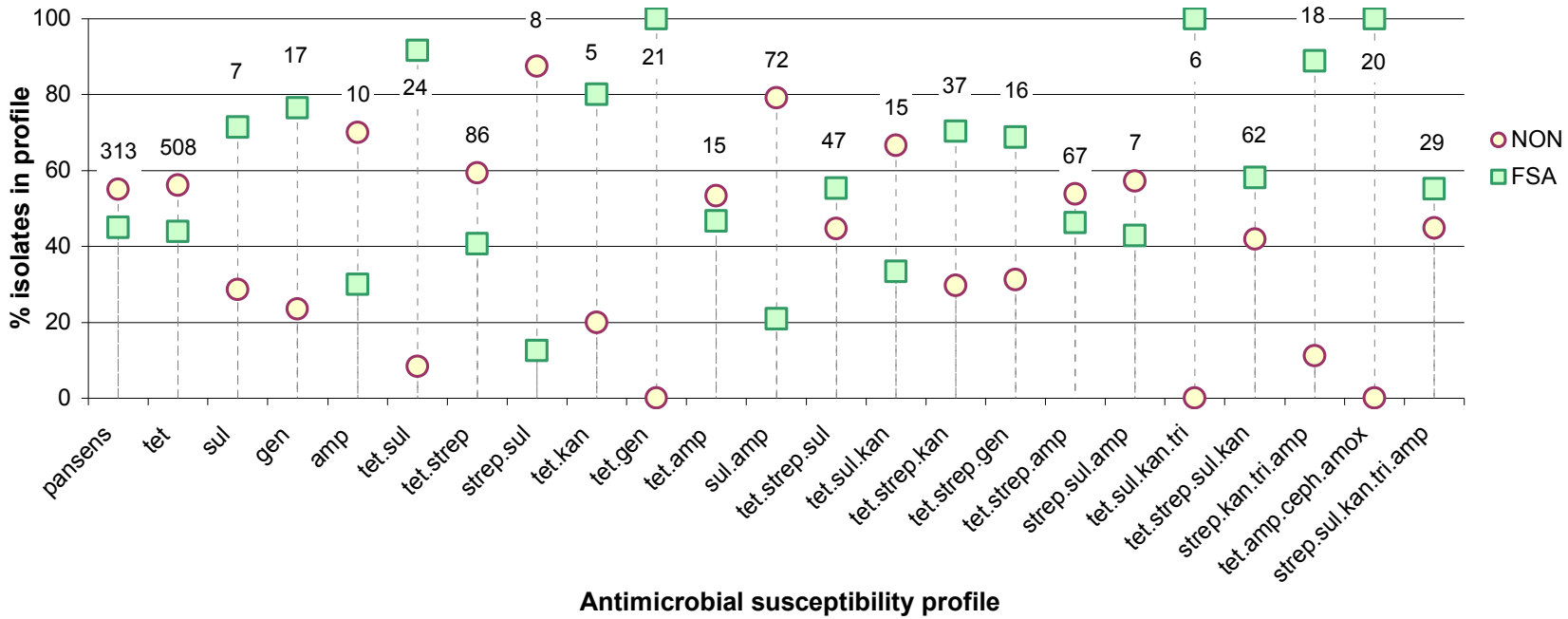
Appendix J. Antimicrobial resistance in *Enterococci* from environmental sources in Trials 1-3, n=36.



Appendix K. Sows: total percent apramycin resistance at 32ug/ml in *E. coli* isolates for Trials 1-3.
Statistical significance: No significant difference between groups over all samples collectively.
Error bars represent 2 standard errors derived from the statistical model.
Note: Scale to 20% only.
Groups: NON=sows of control piglets, FSA=sows of FSA-treated piglets; n=1,981.



Appendix L. Piglets: Sensititre® results from piglet *E. coli* isolates Trials 1-3.
Antimicrobial susceptibility profiles representing 3 or more isolates, 97.4% of total isolates tested, n=1448.
Numbers correspond to total number of isolates from NON and FSA groups in each profile, dashed lines group data by profile.

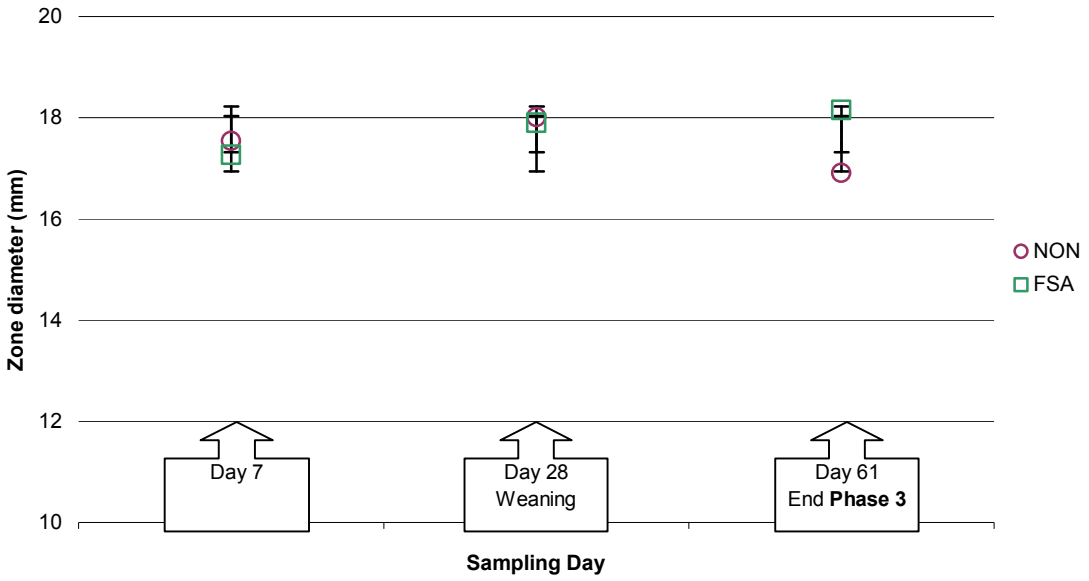


pansens=pansensitive isolates
tet=resistant to tetracycline only
sul=resistant to sulfonamides only
gen=resistant to gentamicin only
amp=resistant to ampicillin only
tet.sul=resistant to tetracycline and sulfonamides
tet.strep=resistant to tetracycline and streptomycin
strep.sul=resistant to streptomycin and sulfonamides
tet.kan=resistant to tetracycline and kanamycin

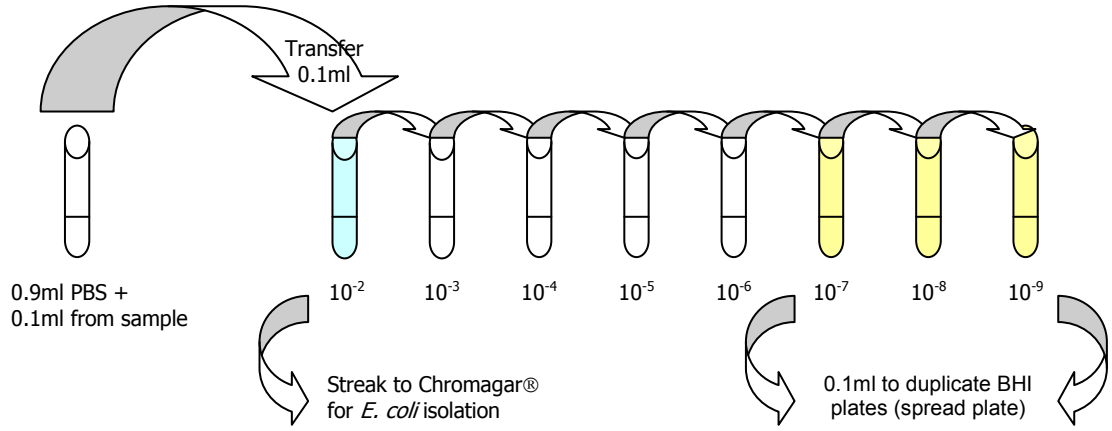
tet.gen=resistant to tetracycline and gentamicin
tet.amp=resistant to tetracycline and ampicillin
sul.amp=resistant to sulfonamides and ampicillin

tet.strep.sul=resistant to tetracycline, streptomycin and sulfonamides
tet.sul.kan=resistant to tetracycline, sulfonamides and kanamycin
tet.strep.kan=resistant to tetracycline, streptomycin and kanamycin
tet.strep.gen=resistant to tetracycline, streptomycin and gentamicin
tet.strep.amp=resistant to tetracycline, streptomycin and ampicillin
strep.sul.amp=resistant to streptomycin, sulfonamides and ampicillin
tet.sul.kan.tri=resistant to tetracycline, sulfonamides, kanamycin and trimethoprim sulfa
tet.strep.sul.kan=resistant to tetracycline, streptomycin, sulfonamides and kanamycin
strep.kan.tri.amp= resistant to streptomycin, kanamycin, trimethoprim sulfamethoxazole and ampicillin
tet.amp.ceph.amox=resistant to tetracycline, ampicillin, cephalothin and amoxicillin
strep.sul.kan.tri.amp=resistant to streptomycin, sulfonamides, kanamycin, trimethoprim sulfamethoxazole and ampicillin

Appendix M. Trial 1 – Carbadox resistance in *E. coli* isolates from piglets, average zones of inhibition. All isolates susceptible at ≥12mm zones (resistant ≤ 10mm).
Note: Scale from 10mm to 20mm.
Groups: NON=isolates from control piglets, FSA=isolates from FSA-treated piglets; n=486.



Appendix N. Serial dilutions of PCE culture for viable plate counts and *E. coli* recovery.



Appendix O. Average daily gains (ADG) for piglets in Trials 4-5.
A trend toward improved gains was noted in PCE-treated piglets.
Groups: NON=control piglets, PCE=PCE-treated piglets; n=132.