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To the Graduate Council:

I am submitting herewith a thesis written by Patricia Cullen entitled "Effects of Management and Environmental Conditions on Antibiotic Resistance in Bacteria Associated with Swine." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Dr. Alan Mathew, Major Professor

We have read this thesis and recommend its acceptance:

Dr. David Golden, Dr. Kelly Robbins

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Dr. Alan Mathew
Major Professor

We have read this dissertation
and recommend its acceptance:

Dr. David Golden

Dr. Kelly Robbins

Accepted for the Council:

Dr. Anne Mayhew
Interim Vice Provost and
Dean of the Graduate School

(Original signatures are on file at the Graduate Student Services Office.)

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Effects of Management and Environmental Conditions on Antibiotic Resistance in Bacteria Associated with Swine

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Patricia Cullen

August 2001

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ABSTRACT

Weaned pigs (n=58) were challenged with nalidixic resistant *Salmonella enterica* serotype Typhimurium and separated into eight treatments to determine the effects of various environmental and management conditions on the development of antibiotic resistance among pathogenic and commensal bacteria. Apramycin sulfate was administered in the feed (150g/ton) two days post-challenge for a period of 14 days with the exception of one control group. Treatments included: control without apramycin (control-1); control with apramycin (control-2); and apramycin plus either cold stress, heat stress, overcrowding, intermingling, poor sanitation, and intervention with oxytetracycline (100 ug / ton). Treatments were applied 5 days post initial antibiotic administration and maintained throughout the study. Fecal swabs were obtained prior to antibiotic treatment (day 0) and on days 2, 7, 14, 28, 64, 148, and 149 post-treatment. *Salmonella* Typhimurium, *Escherichia coli*, and *Enterococcus faecalis* were isolated and tested for resistance to apramycin sulfate, ceftiofur sodium, oxytetracycline, and sulfamethazine via broth microdilution. Increased ($P < .0001$) resistance to apramycin was noted in *E. coli* in all groups administered apramycin by day 14. Control-2 minimum inhibitory concentrations (MICs) returned to baseline following removal of the antibiotic, whereas cold stress, overcrowding, and oxytetracycline groups expressed significantly ($P < .05$) greater MICs through day 64 before returning to baseline. *S.* Typhimurium generally displayed lower MICs for all test antibiotics compared to *E. coli,* while

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E. faecalis demonstrated elevated resistance throughout the study to all antibiotics. These data indicate that *E. coli* resistance to apramycin is significantly increased upon exposure to various stressors, whereas *S.* Typhimurium and *E. faecalis* may be less affected.

Key Words: antibiotic resistance, *E. coli*, swine

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1. INTRODUCTION

Over the past 50 years antibiotic use in the swine industry has become a valuable asset in disease prevention, treatment, and growth promotion (Hays 1986). With the implementation of these products, producers have managed to reduce overall costs, increase pig production turnover rates, and ultimately produce safer, higher quality meats for the consumer (NRC 1999). However, these benefits have recently been compromised by the threat of emerging antibiotic resistant microorganisms. Previous studies have linked the development of resistant enteric bacteria to exposure of subtherapeutic levels of antibiotics (Langlois et al. 1983, Hays 1986, Mathew et al. 1998).

The transfer of resistance factors from one bacterial species to another has compounded this problem by introducing the risk of pathogens acquiring resistance from non-pathogenic organisms. One study reported the transfer of apramycin resistance plasmids from *E. coli* to a pathogenic, as well as, zoonotic organism, *Salmonella* Typhimurium, in calves (Hunter 1991). The potential transfer of these resistance genes from indigenous microflora to pathogens possibly affecting human health has caused a growing concern among animal, and human practitioners. To date, the estimated annual cost of treating antibiotic resistant infections in human health care in the U. S. ranges from $5 billion to as high as $30 billion (NIAID 2000, AAAS 1998). As a result, current research has focused on the examination of potential factors associated with the development of antibiotic resistance in agriculture. For example, various studies have produced comparable results indicating that age of the animal influences levels

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of resistance among bacterial isolates (Hays 1986, Mathew et al.1998). Hays reported tetracycline resistance in 55% of fecal coliforms from pigs younger than 6 months of age compared to 25% in pigs over 6 months of age in an antibiotic-free herd. In addition, isolates from younger pigs, on average, were resistant to more antimicrobial agents than those of more mature pigs (Hays 1986).

Results from other studies also suggest that stress, such as that resulting from transportation of pigs and calves, has an influence on the proliferation of resistant organisms, as well as the excretion of lactose-negative organisms (Hays 1986, Corrier et al. 1990, Langlois et al. 1999). These reports suggest that factors other than exposure to antibiotics may play a significant role in the development of resistant organisms.

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2. LITERATURE REVIEW

Antibiotics in Livestock Production

The introduction of vertical integration and advanced technology to the swine industry has initiated a surge in production (NRC 1999). Trends reveal that the number of hog farms in the U.S. has declined by almost 6% annually from 1967 to 1996, while the number of hogs produced per farm has increased nearly 5 times (Plain 1997). Earlier weaning ages, increased farm capacities, and the incorporation of feed additives to swine diets have allowed producers to further increase productivity. As a result of these intense production systems and potential animal exposure to various environmental and management stressors, concern regarding disease susceptibility has been mounting. The implementation of antibiotics as feed additives in the 1950s has been beneficial to producers from both a production and an economic standpoint in preventing potential disease outbreaks (Cromwell 1991). Since their introduction, antibiotics have had a major impact on livestock production, as indicated in a report revealing the presence of antibiotics in 85%-95% of starter feeds, 75%-80% of grower diets, 55%-60% of finisher diets and 20%-30% of sow diets (Cromwell 1991).

The basic role of antibiotics in the livestock industry is twofold. Aggressive treatment of disease and infection includes the use of high levels of antibiotics and is categorized as therapeutic treatment (NRC 1999). Sub-therapeutic treatment includes the application of moderate levels (<200 g / ton) of

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antibiotics as a means of prophylactic therapy. Additionally, subtherapeutic levels of antibiotics have been added to feeds for increased growth and nutrient utilization in order to meet the high production demands of the market (Jukes 1986, Cromwell 1991).

Benefits of Antibiotics

Through the proper application of antibiotics, producers are capable of generating a high quality product for the consumer, in addition to reducing morbidity and mortality rates within their operations (Cromwell 1991). In this investigation a decrease was detected in mortality rates from 4.3% in young pigs fed a control diet compared to 2.0% in those fed antibiotics (Cromwell 1991). These effects were amplified in a related study evaluating the effects of antibiotics under stressed and "high-disease conditions". Control pigs from this study had a mortality rate of 15.6%, compared to 3.1% in pigs administered antibiotics in the feed (Cromwell 1991).

In addition to improved overall animal health, enhanced feed efficiency associated with the incorporation feed-based antibiotics has also been documented. Hays (1986) reported a 16%, 11%, and 4% increase in average daily gain among starter, grower, and grower-finisher pigs, respectively, that were fed subtherapeutic levels of antibiotics compared to those fed a control diet. The increased effects noted in the starter and grower phases may be attributed to the increased exposure to stress (weaning, environmental changes), as well as the fact that growth rates are higher during these times compared to the

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finishing phase. Although the net effect of treatment diminished with age, the application of antibiotics consistently resulted in performance benefits that initiated an increase in profitability.

Another study evaluating the effectiveness of a feed-additive antibiotic in weaned pigs yielded similar findings (Gorham et al. 1988). Results from this study indicated that after a two-week treatment (150g of apramycin/ton), medicated pigs averaged 1.9 kg heavier than the control pigs, in addition to having a decreased incidence of scours. It is speculated that by reducing the stress on the immune system, increased nutrient levels are made available to the animal, resulting in increased feed efficiency and improved overall animal health (NRC 1999). It should also be noted that the effects of feeding antibiotics have been documented to be negligible when fed to germ-free animals (Novick 1981). It has been proposed that one of the primary effective mechanisms of growth-promoting antibiotics is to decrease the thickness of the small intestine by eliminating microbes and their toxins that adhere to and damage the intestinal lining thereby increasing the potential for optimal nutrient absorption (Grant 1984).

In addition, antibiotics have also been shown to reduce the bacterial catabolism of urea and amino acids; thereby, reducing nitrogen excretion loads into the environment (Corpet 2000).

Aside from growth performance improvements, reproductive benefits in swine from antibiotic feed additives have additionally been realized. Numerous studies have documented improvements in conception rates in sows when fed

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antibiotics at the time of breeding (Cromwell 1991, NRC 1999). Furthermore, improved farrowing rates, litter size, birth weights, and pigs weaned per litter were reported when antibiotics were fed in pre-farrowing and lactation diets (NRC 1999).

By reducing the risk of possible pathogenic organisms infiltrating the U. S. food supply, the use of antibiotics for the above reasons has led to the availability of food products suitable for human consumption. From an economical standpoint, the estimated annual savings in costs to consumers in the United States due to the use of antibiotics was $3.5 billion in 1981 and has most likely increased since then (CAST 1981). Despite the production and economical benefits of incorporating antibiotics into livestock feeds, there has been growing concern regarding the risks involved.

Risks of Antibiotics

Antibiotic resistance had been detected in organisms before the beginning of the antibiotic era; however, recent concern has been growing regarding the substantial rise in the prevalence of resistant organisms (Lewis 1995, NRC 1999). A recent study comparing the incidence of multiple-drug resistant *Salmonella* in 1981 and 1990 supported this theory. Prevalence of multiple-drug resistant *S.* Typhimurium doubled in humans from 1981 to 1988 with an additional 7% increase from 1988 to 1990 (Threfall et al. 1993). Increases in multiple-drug resistant *S* Typhimurium were also detected in cattle, swine, and to a lesser degree in poultry. The lower increase noted in poultry may

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be a result of strong restrictions on the use of antibiotics as growth promoters in that production industry (Threfall et al. 1993).

Various studies have associated the emergence of resistant bacteria with the use of subtherapeutic levels of antibiotics in livestock feeds (Timoney 1978 Langlois et al.1984, Mathew et al. 1998). Langlois et al. (1983) compared the incidence of resistance in fecal coliforms between two herds of pigs: one with no antibiotic exposure within eight years (non-antibiotic herd) and another (antibiotic herd) that had been routinely administered subtherapeutic levels of chlortetracycline (CTC) within the previous eight years. During the study, each of these groups was further divided into a control group (no antibiotic), a subtherapeutic group (fed 27.5 ug/g CTC continuous), and a therapeutic group (fed 220 ug/g for 14 days). Results yielded an elevated mean multiple resistance ($P < .05$) before dosing in the antibiotic herd (3.33) over the non-antibiotic herd (1.64) (Langlois et al. 1983). Subtherapeutic treatment resulted in a greater detection of CTC resistant coliforms within the antibiotic pigs (47%) compared to the non-antibiotic pigs (23%) (Langlios et al.1983). Upon removal of CTC in therapeutic groups, detection of resistant isolates in both the antibiotic and non-antibiotic herds decreased with a more pronounced initial decrease among the non-antibiotic isolates (Langlois et al.1983). Conclusions from this and other studies indicate that fecal coliforms may have a higher potential to develop resistance through continued exposure to feed based antibiotics, in comparison to those organisms with no exposure; therefore, antibiotic therapy may serve as a selection mechanism for the proliferation of resistant bacteria.

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Further information suggests that these indigenous enteric bacteria may also serve as a reservoir for the transmission of resistance factors to various pathogenic and even zoonotic organisms, thus fueling the debate over agricultural use of antibiotics (Smith 1971, Hunter 1991). In the early 1990s a herd of calves was administered a feed based antibiotic, apramycin, to control a *Salmonella* outbreak. Apramycin, an aminoglycoside antibiotic, was first approved in the U. S. for exclusive use in animals in 1986 (Mortensen et al. 1996). It is primarily used for treatment of porcine colibacillosis and bacterial enteritis in cattle associated with weaning stress, or for prevention of those problems via feed/water administration for two weeks (Plumb 1995, Mortensen et al. 1996). In this study detection of apramycin-resistant commensal *E. coli* in feces prior to antibiotic treatment was noted; however, all *Salmonella* isolates expressed sensitivity (Hunter 1991). *In vitro* transfer of resistance from *E. coli* to sensitive *Salmonella* isolates was accomplished in 77 out of 80 occasions in nutrient broth (Hunter 1991). Following antibiotic treatment, i*n vivo* detection of resistance transfer was evident in at least one calf, based upon plasmid profiles of the resistant *Salmonella* and *E. coli* isolates (Hunter 1991).

A similar study involving calves supported these findings regarding *in vivo* transfer of resistance from a high-transmitting strain of *E. coli* F18 to *S.* Typhimurium phage type 29 (Smith 1971). The calves were initially dosed orally with the donor strain followed by the recipient strain 24 h later. Three of the eight calves exhibited no signs of infection and yielded no or minimal numbers of *S.* Typhimurium, all of which were lacking the resistance factors. Resistant *S.*

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Typhimurium were isolated from the intestinal tract of three of the remaining five calves, demonstrating that *in vivo* resistance transfer occurred. These results were comparable to those found in a similar study employing challenge strains of *S.* Typhimurium in chickens, indicating that *in vivo* transfer of resistance is a potential threat for all livestock (Smith 1971).

Based on these results and those from various similar studies, concern regarding the selective pressure of antibiotic administration for resistant indigenous microflora and their ability to confer resistance to pathogenic organisms is warranted (Kasuya 1964, Jarlomen et al. 1969, Timoney 1978, Maine et al. 1999). Consequently, several studies have focused on investigating the nature of these resistance factors, as well as the mechanisms involved in their acquisition.

Genetics of Resistance

Though most of the recent rise in antibiotic resistance has been attributed to the misuse of antibiotics in agriculture and human medicine, evidence suggests that bacterial resistance had been detected in organisms in times preceding the antibiotic era. This is in part because antibiotics are essentially products of microbial synthesis; therefore, those microorganisms synthesizing these products must carry some type of intrinsic resistance (Hays 1986). It is possible that these antibiotic-producing organisms then transfer genetic information coding for resistance to various other species. The basis for transferring and expressing various resistance mechanisms lies in the genetic

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configuration of the organism. A large portion of the emerging resistant organisms acquired the genetic information for resistance through one of two means: chromosomal mutations or plasmid transfer (Israili 1987).

The less common method of developing resistance to antibiotics is a result of spontaneous mutations of single DNA bases located on the bacterial chromosome (Lacey 1984). In cases of chromosomal resistance, genetic information coding for resistance mechanisms is usually present in the bacteria before exposure to antibiotics and may only be transmitted from the resistant organism and its offspring. Studies have shown, however, that this type of resistance is not always permanent (Lacey 1984). An evaluation of chromosomal resistance stability in *Staphylococcus aureus* 1030 mutants illustrated that an average of 79% of isolates lost resistance to rifampicin over a 4 year period of storage at room temperature on agar slants (Lacey 1984). These infrequent mutations are random and occur at the rate of one per million or one per billion cells (Khachatourians 1998).

The remaining majority of resistance development has been attributed to transmissible extrachromosomal DNA, referred to as plasmids (Israili 1987, McClane 1999). Plasmids are small (0.03 to 10% the size of bacterial chromosomes), self-replicating, circular fragments of DNA present in the vast majority of characterized bacteria. In addition to carrying genes for resistance to one or several antibiotics, plasmids may also code for other traits, including toxin production, invasion, colonization, and attachment to intestinal mucosa (Israili 1987). However, a direct relationship between the presence of resistance genes

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and the expression of the virulence factors mentioned above has not been clearly defined. Smith et al. (1979) found neither a significant increase nor decrease in mortality rates for chickens fed resistant and sensitive strains of both *S. Typhimurium* and *Salmonella gallinarium.* Additionally, there was no significant effect on virulence of the resistant strains upon administration of the antibiotic to which the strain carried resistance, when compared to the sensitive strains. However, in-contact chickens (not infected but exposed to challenged birds) experienced heavier and longer durations of excretion of the strain under antibiotic administration (Smith et al. 1979). Conclusions from this study indicate that although virulence factors may not always be associated with resistance, the application of antibiotics in populations containing resistance plasmids may still have substantial effects on disease susceptibility for hosts carrying bacteria that are lacking in these factors.

Plasmids may be incorporated into chromosomal DNA or transferred to other organisms within or outside their species. There are typically three modes of plasmid-mediated resistance transfer in bacteria: conjugation, transduction, and transformation.

The most common method of resistance acquisition is classified as conjugative transfer. This process requires cell-to-cell contact for the transfer of a resistance plasmid from a donor cell to a recipient. Plasmids coding for resistance for one or more antibiotics through conjugation are also referred to as Resistance (R) factors. The second type of resistance transfer, transduction, results from bacterial virus (bacteriophage) transmission. These phages insert

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their nucleic acid core into the host bacterial cell, induce the replication of new virus particles, and ultimately rupture host bacterial cells (Ross 1986). Small fragments of the spliced bacterial DNA may then be incorporated into the phage's own genetic profile. The bacteriophage then proceeds to another organism, where the phage DNA is inserted into a recipient cell's DNA (McClane 1999). If a bacterium harboring genes for resistance to one or more antibiotics is lysed by a bacteriophage, the resistance genes may be easily transferred to another bacterial cell through this process. The third mechanism, transformation, involves the cellular uptake of released, "naked", single-stranded DNA from lysed resistant cells (McClane 1999). This differs from transduction in that there is no vector for transmission. Not all cells are capable of transformation, however. Transformation requires the binding of the DNA to receptors on the surface of the recipient organism, the fragmenting of the DNA by DNAase, and the pairing of the newly formed single stranded DNA with the host DNA. Those cells capable of transforming foreign DNA are classified as competent cells (Ross 1986). The resulting outcome for all methods of resistance acquisition is an ability to utilize the newly acquired genetic coding for various mechanisms involved in antibiotic resistance.

Mechanisms of Resistance

Bacteria utilize the above genetic coding from plasmids to implement various mechanisms necessary for surviving the lethal effects of antimicrobial therapy. Some bacteria, including *Salmonella*, are invasive organisms, able to

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enter intestinal epithelial cells as a means of evading those antimicrobial agents (i.e. aminoglycosides) that are less efficiently absorbed from the intestine (Roof et al. 1992, Prescott 1993).

Upon exposure to an antibiotic, some bacteria may express R factors coding for modifying enzymes; thereby altering the drug's ability to bind, penetrate, or inhibit bacterial growth (Israili 1987). An example of this is seen in bacterial isolates expressing resistance to certain aminoglycosides. Aminoglycosides are relatively broad-spectrum agents directed at inhibiting protein synthesis. Upon entry into the bacterial cytoplasm, these compounds irreversibly bind to the 30S ribosomal subunits and initiate mRNA misreading and ultimately allow an incorrectly charged tRNA to bind to the ribosomal A site (Mortensen et al. 1996, Purdue 1996, McClane 1999). The end result is the synthesis of physiologically ineffective proteins and ultimately cell death; therefore, these drugs are categorized as bacteriocidal. These drugs do have limitations in that they are less effective against gram-positive bacteria and are not readily absorbed across the intestinal cells, making them less effective against invasive bacteria (i.e. *S.* Typhimurium) (Prescott 1993).

Effectiveness of these compounds is further limited in bacteria possessing R factors coding for aminoglycoside modifying enzymes (acetyltransferases, phosphotransferases, nucleotidyltransferases) (Mortensen et al. 1996). These enzymes modify the antibiotic at certain exposed hydroxyl or amino groups (Prescott 1993). For example, the aminoglycoside, apramycin, is inhibited by bacteria expressing the enzyme, type IV aminoglycoside 3-N-acetyltransferase

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(AAC(3)IV). The enzymatic N-acetylation of the drug inhibits the binding of apramycin to the bacterial ribosome, resulting in decreased efficacy of the drug through excretion of the inactivated compound (van de Klundert et al. 1993, Neu et al. 2000). The majority of beta-lactam resistant bacteria share similar mechanisms. Ceftiofur sodium is a third generation beta lactam cephalosporin that inhibits bacterial cell wall synthesis, targeting the formation of the peptidoglycan layer by inhibiting penicillin-binding proteins (PBPs) in proliferating bacteria (Plumb 1995, Neu et al. 2000). PBPs are involved in the crosslinking of polymers to form the peptidoglycan layer in bacterial cell walls (Neu et al. 2000). Resistance factors activate enzymes (beta-lactamases) that alter the drug's affinity for the PBPs by hydrolyzing the cyclic amide bond located within the beta-lactam ring of the drug (Israili 1987). As a result, the drug is rendered inactive and is thus excreted. Antibiotic activity may also be limited by slight alterations within the drug's targeted receptor site. This is also found within beta-lactam resistant bacteria, but to a lesser degree. Under these conditions, PBPs may experience minute alterations in the amino acid sequencing by enzymes referred to as beta-lactamases thereby resulting in a decreased affinity for certain beta-lactam antibiotics (Israili 1987, McClane 1999).

Another means of diminishing antimicrobial activity may be accomplished by limiting access to the drug's target site or sites (outer membrane, cell wall, ribosomes). The lipopolysaccharide layer present in gram-negative bacteria and the thick peptidoglycan in gram-positive bacteria serve as a physical barrier for most antibiotics (Israili 1987, McClane 1999). Hydrophilic antibiotics have

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difficulty penetrating gram-negative bacterial cell walls due to the high lipid concentrations in the outer membrane (McClane 1999). Increased activity of efflux pumps and decreased activity of influx pumps within the bacterial cell are other mechanisms involved in inhibiting antimicrobials' access to their target sites within the cell (Israili 1987). Mechanisms reducing the permeability of an antibiotic have been shown to render antibiotics like oxytetracycline inefficient (Neu et al. 2000).

Oxytetracycline is a broad-spectrum antibiotic that targets the 30S subunit on bacterial ribosomes, impeding aminoacyl-transfer RNA binding to the acceptor site on the mRNA-ribosome complex; a mechanism similar to that of the aminoglycosides (Prescott 1993). However, tetracyclines are bacteriostatic in that they only inhibit cell growth and proliferation, whereas aminoglycosides are bacteriocidal. This is primarily due to their transient binding to the ribosome receptors; therefore, all bacterial functions are returned to normal upon withdrawal of this drug.

Amplification in the production of the antimicrobial target increases the concentration requirement of the drug for optimal efficacy and thus serves as another mechanism for resistance. This is evident in certain bacterial isolates that carry acquired resistance plasmids for sulfonomides (Israili 1987). Sulfonomides inhibit folate synthesis by competitively blocking the conversion of para-aminobenzioc acid (PABA) to dihydrofolic acid (Neu et al. 2000). Sulfonomides generally have a greater affinity for the conversion enzyme, pteridine synthase, than PABA in this reaction (Neu et al. 2000). Resistant

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bacteria undergo hyperproduction of PABA; thereby, increasing the sulfonomide concentration requirement for optimal efficacy.

Regardless of the mechanism involved, much of the recent concern focuses on the fact that resistance plasmids have been reported to transfer resistance across bacterial species and genus, both *in vitro* and to a lesser degree *in vivo,* as mentioned previously (Smith 1979, Hunter 1981). Moreover there have been documented reports of transfer of resistant microorganisms from farm animals to food handlers and even consumers (NRC 1999). Under "normal"' situations these plasmids may not always be expressed; however, upon induction of abnormal situations (exposure to a particular antibiotic or stress) R factors may be expressed for survival purposes (Lacey 1984).

Impacts of Stress

While there is no precise scientific definition of the constituents of *stress* as it pertains to animals, it has generally been portrayed as an "internal manifestation" of adverse influences (psychological, physiological or environmental) affecting the homeostasis of an individual (Roth 1985, Peterson et al. 1991). As a result of the rise in intense swine production systems, the potential exposure to various environmental and managerial stressors has caused much concern regarding disease susceptibility and antibiotic resistance. Increased farm capacities, mass transport and mixing, and variations in environment and management may force animals to adapt through various physiological responses to compensate for abnormal conditions.

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Upon exposure to various stressors the hypothalmic-pituitary-adrenal axis is activated via CNS innervation (Young 1981, Roth 1984). Stimulation of the hypothalmus leads to the release of corticotropin-releasing hormone (CRH), which in turn activates the release of adrenocorticotropin hormone (ACTH) by the anterior pituitary gland (Guyton 1996, Hicks et al. 1998). ACTH is responsible for the release of the adrenocortical hormone, cortisol, from the adrenal cortex into the peripheral circulation. (Wallgren et al. 1994, Hicks et al. 1996). The effects of these responses have been reported to result in a diminished immune response, a reduction in growth performance, and an alteration in gastrointestinal activity among other things (Owen et al. 1983, Wallgren et al. 1994).

While several studies have linked the influence of various stressors to the overall immune status of the animal, findings concerning the net effect on the animal have often been contradictory (Blecha et al. 1981, Pohl et al. 1983, Tuchsherer et al. 1998). This could be attributed to the fact that a number of factors are involved in determining the level of response initiated by the immune system (i.e. types of stress, intensity, duration, status of the animal, environment, and immune the parameters measured) (Tuchsherer et al. 1998). The effects of hierarchy establishment in mixing pigs resulted in an immunostimulatory response in dominant pigs while an immunosuppressive response was found in subordinate pigs (Tuchsherer et al. 1998). Further conclusions from this study targeted cell mediated immunity (T cell proliferation) as the primary immune parameter affected by mixing.

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The suppressive effect of cortisol on lymphocyte proliferation has been well documented (Morrow-Tesch et al. 1994, Franci et al. 1996, McClane 1996, Tuchsherer et al. 1998). Wallgren et al. (1983) supported this theory by administering an ACTH injection to measure lymphocyte production in stress-simulated conditions in swine. A significant decrease in lymphocytes associated with a substantial rise in plasma cortisol concentrations was noted. Upon withdrawal of the ACTH administration, lymphocyte numbers returned to baseline. Neutrophilic granulocytes, however, increased significantly following elevated levels of plasma cortisol concentrations, indicating the implementation of a nonspecific mechanism for protection.

Pohl et al. (1999) found slightly contradictory results in the evaluation of the effects of thermal stress on the immune response in feeder pigs. Results from that study indicated a significant suppression of both B and T cell response among cold stressed (10°C) animals compared to those in the control group (21°C). In contrast, heat stressed (32°C) animals exhibited higher T cell responses and lower B cell responses than the control, demonstrating that immunostimulatory effects may also be associated with certain types of stress induction. Another study illustrating the immunoenhancement effects of acute stress showed an increase in antibody titers occurred upon the exposure of weaned pigs to cold stress (Blecha et al. 1981). It has been suggested that acute stress may often stimulate immune responses, while chronic stress tends to have detrimental effects on immunity (Griffin 1989). This reallocation of

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leukocytes stimulated by cortisol release poses a risk to stressed animals in relation to disease susceptibility.

Evidence of this theory was supported by a report noting an increased incidence in *Salmonella* in horses stressed by transportation (Owen et al. 1983). Furthermore, detection was prolonged upon administration of oxytetracycline in addition to transport. Similar stress studies involving cold stressed mice documented increased mortality among stressed subjects following exposure to *Staphylococcus aureus* or *S.* Typhimurium, while control animals maintained normal health status (Miraglia et al. 1962, Previte et al. 1962, Edwards et al. 1977). The effects on humoral and cell-mediated immunity was may be demonstrated in one particular study where susceptibility of a secondary Staphylococci infection was increased following a challenge with *Salmonella* (Miraglia et al. 1962).

Stress-mediated alterations of the immune system require the repartitioning of energy away from the maintenance processes of growth and metabolism. Elevated blood cortisol concentrations have been associated with stimulation of gluconeogenisis, fatty acids mobilization, amino acid mobilization, and anti-inflammatory responses to compensate for the energy reallocated for managing stress (Roura et al. 1992, Guyton 1996). Introduction of chronic unsanitary conditions in broilers resulted in significantly lower growth rates and feed efficiencies among stressed birds compared to those raised in clean environments (Roura et al. 1992). Feed efficiencies and weight gain were significantly greater in birds exposed to unsanitary conditions when birds

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received antibiotics compared to birds kept in similar conditions without antibiotic exposure. Similar consequences have been documented for overcrowding, through simulations of intensified swine production systems, during starter, feeder and finisher phases (Harper et al. 1983, NCR 1984). Conclusions from these studies indicate that the effects of chronic environmental stress on growth are detrimental, but may be alleviated via antibiotic therapy. It is during these and other “high risk situations” such as weaning, shipment, or severe weather that the prophylactic administration of antibiotics may be beneficial (Gustafson 1986).

Other manifestations of the effects of stress may include changes in gastrointestinal activity. It has been suggested that stress-induced CRH release may also be responsible for decreased gastric acid secretion, gastric emptying, and inhibition of small intestinal motility (Lenz et al. 1988). Additionally, studies indicate that stress may also enhance colonic transit and fecal excretion (Lenz et al. 1988, Barone et al. 1990). The activation of the parasympathetic stimulation in the colon releases the neurotransmitter, acetylcholine, which in turn induces smooth muscle contractions in the colon (Barone et al. 1990, Guyton 1996). This increased propulsion of material from the intestinal tract of a stressed animal has been associated with an increase in fecal shedding of a diverse population of microbial organisms. Indigenous microflora create a unique ecosystem, in which various species interact with each other under normal gut conditions. When stress compromises these conditions (i.e. altering pH, temperature, and gut atmosphere), selection for certain species disturbs the microbial population, thus

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allowing for potential colonization of pathogenic organisms (Thayer 1987, Moro 1996). Corrier et al. (1990) reported an increased excretion of various *Salmonella* species associated with marketing and transportation stress in feeder calves. More significantly, all isolates recovered exhibited resistance to five or more antibiotics. Similar results were found in a herd of pigs with no previous exposure to antimicrobial therapy. Initial fecal samples were taken under normal conditions, again at a loading area, immediately following a 30-minute transport and 24 hours post transport. Samples taken at the loading dock and immediately following transport yielded significantly higher incidences of resistance in gram-negative organisms than those from unstressed pigs (Langlois et al. 1999). However, samples taken 24 hours post-transport revealed resistance among stressed isolates returned to baseline values, indicating that resistance was of transient nature. Therefore, under certain stressful situations enhanced and selective excretion of resistant bacteria may be linked to an altered genetic coding in resistance plasmids. It may be possible that the genes coding for resistance are somehow linked to a gene controlling adhesion factors. Therefore, those resistant organisms may be less inclined to adhere to the colon during stress-induced mass movements.

It has been noted that induction of stressors at the farm level not only diminishes the immune system and growth performance of livestock, but also enhances the possible selection of resistant organisms. Concern among health care specialists focuses on the possibility of these organisms infiltrating the human food supply and contributing to the rise in bacterial infections that are

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unresponsive to antimicrobial therapy. In order to prevent the promotion of bacterial resistance, the demand for further information regarding the association between environmental and management stressors and development of resistance in microbial populations is warranted.

Enterococcus faecalis

Enterococcus faecalis is a gram-positive indigenous microorganism found within the intestinal tract of man and animals. Despite their low virulence these organisms have been identified as opportunistic pathogens in immunocompromised hosts (Moellering 1998). Recent reports suggest *E. faecalis* is the predominant member of the *Enterococcus* genus associated with human infections, accounting for 79-90% of enterococci clinical cases (Huycke et al. 1998, Moellering 1998, SAARS 2000). Their high resistance to a wide array of antimicrobial products plays a significant role in allowing for their selection in nosicomal infections. Resistance outside of hospital settings has also been reported. A study evaluating the prevalence of resistance in environmental samples indicated a substantial rise in intensity of acquired resistance to aminoglycosides, known as high-level aminoglycoside resistance (HLAR) (Rice et al.1995). Furthermore, *E. faecalis* has been documented to transfer resistance genes to organisms from other bacterial genuses by means of conjugation (Haack et al.1995).

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Eschericia coli

Gram-negative, commensal *Escherichia coli* has also been established as a reservoir for antimicrobial resistance genes (Hunter 1992). Resistance to tetracycline, a common antibiotic used for growth promotion in swine production, has been reported to be as high as 71- 90% in *E. coli* isolates from finisher swine (Molitoris et al. 1987, Dunlop et al.1998). Such resistance presents risks to successful treatment of diseased pigs in stressful conditions such as at weaning and transport. If resistant *E. coli* transfer resistance factors to targeted pathogenic organisms, antibiotic treatment may be rendered ineffective against those organisms as well.

Commensal bacteria, while helpful in controlling infections through stimulation of the immune system and competitive inhibition of pathogens, may also have serious detrimental effects regarding the maintenance and spread of antibiotic resistance to pathogenic organisms.

Salmonella Typhimurium

Salmonellosis has been identified as one of the most costly foodborne pathogens in the U. S. A., responsible for at least 50,000 documented cases and registering an estimated $0.69 to $3.8 billion in medical expenses annually (McClane 1996, Isaacson et al. 1999). However, a vast majority of *Salmonella* gastroenteritis cases often go unreported; therefore, the actual number of cases each year may extend into the millions (McClane 1996). Porcine salmonellosis alone accounts for $100 million in production costs nationally (Roof et al. 1992).

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The high costs and difficulties found in treating this disease may be attributed in part to the invasive nature of this organism. S*almonella enterica* serotype Typhimurium, one of the predominant serovars of this pathogenic organism, is a gram-negative, flagellated, facultative anaerobe commonly found within the gastrointestinal tract of infected swine, cattle, poultry, and man (Roof et al. 1992).

Transmission of this organism generally occurs through a fecal-oral route of a relatively high infective dose (10^6 – 10^{11} CFU/ml) (Roof et al. 1992, McClane 1996). Upon ingestion, *Salmonella* pass through the stomach and colonize primarily in the ileal portion of the small intestine (Roof et al.1992, McClane 1996). A crucial factor in establishing an infection is the organism's ability to adhere to and permeate the host's intestinal epithelium (Isaacson et al. 1992, Mclane 1996). Invasion of enterocytes and membranous cells located on the Peyer's patches and stimulation of humoral and specific immune responses results in diarrhea, abdominal cramping, fever, nausea, vomiting, and in immunocompromised subjects, death (Abbas et al. 1997, Procyk et al. 1999). These effects are frequently enhanced during stressful conditions (i.e. weaning, crowding, and transportation) in swine production (Roof et al. 1992, Isaacson et al. 1999).

Zoonotic spread of *Salmonella* has been documented primarily through the food chain (contaminated meat); however, a secondary route of infection between livestock and farm and processing plant personnel has been identified as well, thus causing concern among healthcare specialists (Novick 1981, Holmberg et al. 1984). Adding to the complexity of the situation, swine with

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chronic *Salmonella* infections that are asymptomatic become subclinical carriers, making detection more difficult at the slaughtering plant (Isaacson et al. 1999).

Administration of antibiotics has been shown to reduce the incidence of *Salmonella* in swine, although simultaneously increasing the number of resistant isolates (Ebner et al. 2000). Moreover, Holmberg et al. (1984) linked contaminated hamburger meat originating from beef cattle administered subtherapeutic levels of chloramphenicol for growth promotion to an outbreak of human salmonellosis. It has also been suggested that human use of antibiotics in a portion of the cases led to the selection of resistant organisms and clinical expression of possible asymptomatic infections (Holmberg et al. 1984). Findings from this report were comparable to another human outbreak of *Salmonella enterica* serotype Newport involving hamburger from a dairy farm administering subtherapeutic levels of chloramphenicol (Spika et al. 1987). The ability of pathogenic *Salmonella* to transfer resistance genes similar to these to indigenous *E. coli* broadens the pool of resistant organisms threatening human health (Timoney 1978).

Another aspect of significant clinical importance is the emergence of a particular strain of *Salmonella*, *Salmonella* Typhimurium DT104 that exhibits resistance to as many as five antimicrobials including, ampicillin, chloramphenicol, streptomycin, sulfonomides, and tetracycline (R-type ACSSuT) (Angulo 1997). Resistance of this organism has been found to be chromosomally integrated, allowing for prolonged resistance regardless of antibiotic withdrawal (Threfall et al. 1994). First isolated in the United Kingdom in

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1984, detection of DT104 in humans and animals has been reported in the United States, Denmark, Germany, France, and Canada (Angulo 1997). Annual estimated economic costs from DT104 in the United States range from $67-$900 million (Akkina et al.1999). Prevalence of DT104 in humans has increased significantly (0% in 1980; 14% in 1985; and 38% in 1990) as noted in a study testing R-type ACSSuT *S.* Typhimurium isolates from 11 states in the U. S. (Glynn et al. 1998). Possible risk factors associated with the spread of pathogenic organisms similar to DT104 are antibiotic exposure and exposure to various stressors (overcrowding and transport) (Akkina et al. 1999). These findings emphasize the need to implement proper management conditions and prudent use of antibiotics at the farm level, as well as in human medicine in order to minimize the spread of antibiotic resistance.

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3. MATERIALS AND METHODS

Animals, Housing, and Treatments

Fifty-eight pigs (18 days old) with no history of antibiotic exposure were obtained from the University of Tennessee Blount Swine Research Station for this analysis. Pigs were weaned and transported to the Johnson Animal Research Teaching Unit (JARTU) for the conduction of the experiment.

Upon arrival at JARTU, all pigs were challenged intranasally with 10^{11} CFU of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (National Animal Disease Center, Ames, Iowa), containing a nalidixic acid resistance marker for selected detection of the challenge organism. Inoculum was prepared from select colonies incubated 24 h at 37°C on XLT4 agar (Difco, Sparks, MD) containing 50 ug/ml of nalidixic acid (Sigma, St. Louis, MO). Colonies were transferred to flasks containing 200 ml Nutrient Broth (Becton Dickenson Sparks, MD) and incubated 18 h at 37°C in a shaking incubator.

Pigs were randomly assigned to one of eight separate treatment rooms summarized in Table 1(All table and figures are located in the Appendix). All groups were managed under optimal housing conditions according to NRC recommendations with adjustments for stressed treatments. Lighting for all rooms consisted of 12 h of light and 12 h of dark. *Ad libitum* access to feed and water was applied to all treatments. Diets were formulated for starters and feeders/growers, as represented in Tables 2 and 3.

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The control group (Control-1) received no exposure to the feed-based antibiotic, apramycin sulfate (ApralanType B Elanco Animal Health Division of Eli Lily) and was housed under optimal conditions. Control-2 pigs were raised under identical conditions but fed subtherapeutic levels of apramycin (150g/ton for 14d) in their diet. The same antibiotic concentration was administered to all stressor treatments for the allotted time period. A thermal deficit of 6°C was maintained in the cold stress treatment compared to the Control-1. Similarly, an elevation of 6°C from the Control-1 group was sustained in the heat stressed room. Relative thermal adjustments for growth were made for all rooms throughout the study (NPPC 1996). Oxytetracycline pigs were administered subtherapeutic levels of oxytetracycline (100g/ton TM-50 Type A Pfizer Inc.,Exton, PA), in addition to apramycin upon initiation of treatments and continuing throughout the study. The poor sanitation room was cleaned on a monthly basis, in contrast to the Control-1 cleaning regimen of three times a week. Pigs in the overcrowding treatment were held at a 30% reduction in floor space. To accomplish this, 10 pigs were placed into a typical nursery pen compared to the standard 6 pigs. Adjustments in pen size according to growth maintained crowding conditions in finisher pens. In the intermingling treatment, six pigs received apramycin and were allowed nose-to-nose contact and fecal exchange with adjacent pens containing a total of 6 additional pigs with no previous exposure to apramycin.

All rooms were cleaned and sanitized thoroughly prior to the study. Proper biosecurity precautions were taken before and after entrance and exit from each room. During feeding and cleaning, authorized personnel wore

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disposable coveralls (Fisher, Suwanee, GA), gloves (Microflex, Malaysia), and plastic boots (Nasco, Ft Atkinson, WI), changing coveralls and gloves between rooms. Foot baths were used before and after entrance and exit from rooms, respectively. Rooms were cleaned three times per week with the exception of the crowding and poor sanitation treatments. The overcrowding room was cleaned three to four times a week to simulate a similar sanitation condition as the control without apramycin.

Pigs were housed in nursery crates (4' X 4') for one month at which point they were transferred to elevated (6"), grated finishing pens (8' X 8', with the exception of the overcrowding treatment, which was adjusted accordingly) located in the same treatment rooms. Each group was administered apramycin, two days post-inoculation (day 2), with the exception of the Control-1 group. Apramycin administration was continued for 14 days (maximum label use) according to recommendations for the prevention of colibacillosis (Gorham et al. 1988). Management and environmental treatments were applied seven days post-challenge (day 7) to allow for acclimation. At the end of the study (day 148) one half of the pigs from each treatment were mixed and transported to a common holding facility (Plateau Experiment Station, Crossville, TN) approximately one and a half hours away to test for effects of mixing and transport on antibiotic resistance. Fecal samples were taken prior to and 24h after transport (day 149) and processed accordingly.

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Sampling and Microbiological Analysis

Two fecal swabs (Fisherbrand Dacron Sterile Swabs, Houston, TX) were taken from each pig prior to inoculation (day 0) and again on days 2, 7, 14, 28, 64, 148 (prior to shipping), and 149 (post-shipping) for the recovery of *S.* Typhimurium, commensal *Escherichia coli*, and commensal *Enterococcus faecalis*. A maximum of 48 bacterial isolates of each organism were taken from each treatment group. Swabs were then transported in sterile glass tubes (60 X 150 mm, Fischer Scientific, Pittsburgh, PA) on ice to the laboratory for microbiological analysis.

For the isolation of *E. faecalis* one fecal swab tip from each pig was placed in stomacher bags (Seward Model 80 Tekmar, Cincinnati, OH) containing 80ml of Enterococcosel Broth (Becton Dickenson, Sparks, MD) for 24h at 35°C for enrichment. Sodium azide serves as the selective agent for gram-positive bacteria in this media. Hydrolysis of esculin in the presence of bile, which is characteristic of enterococcus, is indicated by a change in color of medium from brown to black.

From this culture 10ul were transferred to Steptosel Agar plates (Beckton Dickinson, Cockeysville, MD) containing 0.04% potassium tellurite, as a selective agent for *E. faecalis*, (Sigma, St. Louis, MO) and incubated at 35°C for 48h. APIStrep strips (Vitek bioMerieux, Syosett, New York) were used for a series of biochemical tests for the confirmation of randomly selected bacteria.

The second swab was streaked onto lactose MacConkey agar (Difco, Sparks, MD) and incubated for 24h at 37°C for the isolation of *E. coli*. Isolates

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were transferred to Trypticase Soy Agar plates containing 5% defibrinated sheep blood (Beckton Dickinson, Cockeysville, MD) and incubated for another 24h at 37°C to select for non-hemolytic colonies characteristic of enterotoxigenic *E. coli* (Gorham et al. 1988, Hampson et al. 1985).

The second swab was placed in 2 ml of Mueller-Hinton II cation adjusted broth (MH II) (Becton Dickenson, Sparks, MD). The tip of the swab and 1 mL of broth was transferred to a stomacher bag containing 80ml of tetrathionate broth (Difco, Sparks, MD) and incubated at 42°C for 24h for enrichment of *S.* Typhimurium. Ten microliters of the tetrathionate culture were plated onto XLT4 agar, containing 50ug/mL nalidixic acid, and incubated for 24h at 37°C. Biochemical confirmation of the challenge organism was completed upon incubation of suspect colonies at 37C on Triple Sugar Iron (TSI) (Difco, Detroit, MI) and Lysine Iron Agar (LIA) (Difco, Detroit, MI) slants (McClane 1996).

Confirmed isolates of *S.* Typhimurium in TSI slants yielded acidic reactions (yellow) in the butts of tubes (indicating glucose fermentation), alkaline slants (red), and hydrogen sulfide production (black) (McClane 1996).

LIA media tests for the decarboxylation of lysine, fermentation of sugars, and production of hydrogen sulfide. Inoculation of *S.* Typhimurium into LIA slants results in an initial acidic reaction (fermentation of glucose), which is reversed by the rapid decarboxylation of lysine, resulting in a purple (alkaline) tube (Difco Manual 1984). Black coloring throughout the tube (hydrogen sulfide production) is indicative of another characteristic of *S.* Typhimurium. The hydrogen sulfide

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produced from sodium thiosulfate reduction reacts with the ferric ammonium citrate to generate a blackening of the media (Difco Manual 1984).

Antibiotic Resistance Testing

Confirmed bacterial isolates were tested for sensitivity to apramycin sulfate (Sigma, St. Louis, MO), ceftiofur sodium (Naxcel, Pharmacia & Upjohn Co., Kalamazoo, MI), oxytetracycline (Sigma, St. Louis, MO), and sodium sulfamethazine (Sigma, St. Louis, MO) via minimum inhibitory concentration (MIC) broth dilution method according to National Committee for Clinical Laboratory Standards (NCCLS). For this analysis, bacterial isolates were grown individually to a McFarland standard of 0.5 ($\approx 10^8$ CFU/ml) in 5ml MH II broth cation adjusted (Becton Dickenson, Sparks, MD) at 37°C for *E. coli* and *S.* Typhimurium and 35°C for *E. faecalis* (NCCLS 1997). Once adjusted to required concentration, 25.3ul of culture was transferred to 2.5ml of a 1:10 dilution mixture of sterile water and MH II broth. Fifty microliters of this solution were then promptly transferred to microtiter trays for analysis, resulting in a final bacterial concentrations of approximately 5×10^5 CFU/mL, as recommended by NCCLS (1997). Microtiter plates consisted of twelve columns and eight rows. The top row was loaded with 50ul of MH II and a solution of one of the four antibiotics. Two-fold serial dilutions were carried down the rows, leaving the final row without antibiotics to serve as a control. The twelfth column was reserved for a control strain for each bacterium tested (i.e. *E. coli* "coast" ATCC 29922, *S.* Typhimurium 798 4232, *E. faecalis* ATCC 29212), of which known MIC values

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had been reported. Breakpoints for antibiotics (referenced from NCCLS) and antibiotic dilution ranges for each bacteria species are listed in Table 4.

Recovery of *Salmonella* Typhimurium via Necropsy

Recovery of *S.* Typhimurium dropped significantly after week 9 (day 64), and was undetectable in oxytetracycline group for the remainder of the study. An attempt to recover isolates from internal organs (palantine tonsils, duodendum, duodenal contents, jejunum, jejunal contents, ileum, ileal contents, colon, colonic contents, spleen, cecum, cecal contents, mandibular lymph nodes, and colonic lymph nodes) of a randomly selected pig in the oxytetracycline treatment was conducted 3 months post-challenge, according to methods previously described by Wood et al. (Wood 1992). Samples were enriched in tetrathionoate broth, as well as in selenite cysteine broth, and plated as previously described for detection of *S.* Typhimurium.

Re-inoculation of *Salmonella*

Continued failure to recover *S.* Typhimurium, led to an attempt to re-inoculate pigs from the oxytetracycline group using an isolate from previous sample in that treatment. Preparation of inoculum was prepared identically to the initial inoculation with the exception that the isolate originated from the oxytetracycline group. A dose of 10^9 CFU/ml was intranasally administered to each pig within the group. Pigs from this group were sampled two and three

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days following re-inoculation, producing positive results; however, isolates of *S.* Typhimurium were not detected on the following scheduled sample dates.

Statistical Analyses

A completely randomized design with replication was used to compare the eight treatments (control, control with apramycin, cold stress, heat stress, overcrowding, intermingling, low sanitation, and oxytetracycline). Each pen consisted of six pigs, with each pig representing an experimental unit. Analysis of variance was conducted using mixed model procedures to determine the effects of treatments and interactions of time by treatment (8 samplings) and mixing and transport (SAS 1997). Least squares means were computed and compared using a least significance difference at P = 0.05. Sensitivity to antibiotics and the number of resistant isolates from each treatment group were compared using least squares means estimates of the linearized breakpoints (i.e. if MIC was <2 then=0; if 2 then=1; if 4 then=2; etc) (SAS Mixed Procedure, SAS 1999). Unequal variances were allowed when necessary. Percentage of resistant organisms and multiple resistance was determined using Proc Freq SAS.

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4. RESULTS

E. coli

Results from the *E. coli* data illustrated the most pronounced effects of stressors on the development of antibiotic resistance. Control-1 isolates exhibited the lowest resistance throughout the study, indicating biosecurity between rooms was maintained throughout the study. Significant ($P < .0001$) Treatment effects and Time differences within treatments were noted with resistance to apramycin (Figures 1 and 2). Peak resistance developed in remaining rooms by day 14. Upon withdrawal of apramycin, control-2 levels of resistance returned to baseline levels, whereas stressed groups maintained greater MIC values through day 28 ($P < .05$). Cold stress, overcrowding, and oxytetracycline treatments demonstrated higher MIC values for as long as day 64 before returning to baseline. Although there was a slight increase in MIC values in all groups following transport with the exception of control-1 and the poor sanitation, post transportation MICs were not significantly different from pre-transportation levels. *E. coli* remained susceptible to ceftiofur in all treatment groups throughout the study.

Time differences within treatments, as well as Treatment effects, were noted for ceftiofur, oxytetracycline and sulfamethazine ($P < .05$) (Figures 3-8). Although Treatment as well as Time differences within treatments were noted for ceftiofur, all isolates remained sensitive to that antibiotic throughout the course of the study.

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High levels of resistance to oxytetracycline (beyond the range of our detection) were exhibited by the majority of isolates from the beginning of the study; therefore, no enhanced effects of treatment application were measured. There was a general increase in MICs for sulfamethazine as the study progressed, with a slight decline between days 148 and 149 for all treatment groups.

Salmonella Typhimurium

S. Typhimurium was recovered from most pigs between days 0 and 64, with concentrations declining after day 28. Throughout the study *Salmonella* isolates remained susceptible to apramycin and ceftiofur with no significant treatment effects being noted, whereas effects were detected for oxytetracycline and sulfamethazine (Figures 9-16). Time differences within treatments ($P < .05$) were noted with apramycin, ceftiofur, oxytetracycline and sulfamethazine. In all treatments throughout the study *Salmonella* remained susceptible to apramycin and ceftiofur, demonstrating only slight variations in MICs.

MICs for oxytetracycline in the group administered oxytetracycline demonstrated a general rise in resistance after application of that antibiotic and continued through day 28. Unfortunately, inability to detect *Salmonella* at later sampling dates hindered further evaluation of this potential trend. Isolates from heat, cold, intermingling and the control with apramycin pigs remained susceptible throughout the study. Poor sanitation pigs yielded a general decline in MICs throughout the test period. Interestingly, isolates from the control without

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apramycin generally exhibited the greatest MICs throughout the study; however, resistant organisms were traced back to a single pig in the majority of the cases.

Resistance to sulfamethazine increased steadily among isolates from the crowded pigs, whereas all other treatments exhibited a reduction of resistant isolates by day 14 followed by a general increase that was maintained until detection of *Salmonella* ceased. Effects of transport on *Salmonella* resistance could not be determined due to lack of recovery of the challenge organism beyond the day 64.

Enterococcus faecalis

Despite numerous attempts to recover *E. faecalis*, detection immediately following antibiotic administration and stressor initiation (days 7 and 14) dropped substantially, but returned to original levels by day 149. Data in Figures 17-24 reflect the MICs of isolates recovered from the eight treatment rooms. Time differences within treatments ($P < .05$) were observed for all treatments when tested against apramycin and ceftiofur, while Treatment effects ($P < .05$) were noted for all antibiotics. A complete analysis of resistance trends was not possible due to the lack of recovered isolates on days 7,14, and 28, which were critical dates for detecting resistance development among *E. coli*. The dependability of results produced for these sample periods was questionable due the low recovery rates; consequently, no conclusions or inferences have been made. High levels of resistance to apramycin (>500 ug/ml) were detected for cold, crowding, and oxytetracycline groups on day 148, whereas remaining

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treatments exhibited resistance at lower levels throughout the study. Although varying in degrees, *E. faecalis* isolates generally exhibited resistance to all antibiotics throughout the study.

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5. DISCUSSION

Controlled studies evaluating the effects of various environmental stressors on the development of antibiotic resistant bacteria from pigs have been limited due to the difficulty in controlling the numerous influential factors associated with their natural environments. This study was designed to evaluate the effects of certain stressors associated with swine production systems on antimicrobial resistance by altering specific factors within a controlled investigation.

Salmonella Typhimurium

Detection of the challenge organism from some pigs just prior to challenging may be explained by the method in which pigs were inoculated. Following treatment assignments, each pig was sampled, challenged intranasally, and tagged, respectively. Only those pigs that were processed last in each treatment group yielded isolates of our challenge strain that was marked with nalidixic acid for recovery purposes. It is possible that upon placing the processed pigs back into the pen, those pigs may have come into direct contact with the pigs that had not yet been processed.

It should also be noted that those isolates detected on day 0 demonstrated substantially higher levels of sensitivity to oxytetracycline and sulfamethazine than any other sample date throughout the study. This may indicate that the challenge organism underwent a change in resistance expression upon exposure to animal tissue or the ambient environment. This

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expression may have been induced by the organism's environmental change from that of a culture medium to a physiological atmosphere.

All pigs exhibited yellow watery diarrhea 24 hours post-inoculation, which has been identified as a primary clinical sign of infection with *S.* Typhimurium (Roof et al. 1992). Although clinical signs ceased after day 2 post-challenge, detection of *Salmonella* isolates up to day 64 indicated that pigs maintained a carrier status throughout the majority of the study. Although to a slightly lesser degree, the duration of *Salmonella* excretion is reflective of a past study, documenting loss of detection after day 70 (Ebner et al. 2000). However, that study was conducted on a farm that had been in existence for over thirty years, whereas this experiment was conducted at a fairly new research center built less than 3 years ago. A similar study also conducted at JARTU reported loss of *Salmonella* detection 8 weeks post-challenge, which is comparable to our loss at 9 weeks (Jackson 2000 unpublished). Duration of infection has been reported to be dependent on host environment, adhesiveness of the organism, uptake of the organism by host cells, and release into intestines; therefore, it was unusual that detection of *Salmonella* dropped in the poor sanitation room within the same time period as the other treatments (Isaacson et al. 1992). This may be attributed to the fact that pens were raised 6 "from the ground, reducing the possibility for re-inoculation via fecal-oral route. It is believed that animals in this room were stressed nonetheless, due to the increased numbers of flies and feces.
Although a previous study detected the persistence of *Salmonella* in the internal organs in swine up to 28 weeks, our attempt to recover the challenge organism

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from the lymph nodes, tonsils, and intestinal lining after day 64 failed, indicating that pigs had eliminated the organism from both its gastrointestinal and other systems (Wood et al. 1989). Contrary to previous findings, the application of stress had no significant impact on duration of shedding.

The lack of detection of *Salmonella* in the later stages of the study prevented the analysis of effects of transportation on antimicrobial resistance development. Although the stress of transport has been associated with increased excretion of *Salmonella* from pigs in carrier states (Corrier et al. 1990, Isaacson et al. 1992), this was not evident in our study. Complete elimination of the challenge organism from the bacterial pool may have contributed to this result, despite the increase in gut motility combined with other effects of stress (gut pH alteration, immune suppression) that would normally promote excretion of foreign organisms. Based on findings from previous studies, we expected to detect an increase in resistance levels following transportation had we been able to isolate *Salmonella* following transportation (Langlois et al. 1999, Corrier et al. 1990).

The high levels of sensitivity for *E. coli* and *Salmonella* to apramycin and ceftiofur throughout the study in comparison to those found in oxytetracycline and sulfamethazine may be attributed to the combination of the widespread use of the latter two antibiotics in the livestock industry and acquired resistance (Prescott 1993). Both apramycin and ceftiofur are relatively new drugs approved for use in the 1980s and 90s respectively, whereas oxytetracycline and

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sulfamethazine have been routinely used for growth promotion and disease treatment and prevention since the 1950s (Nichols 1991, Mortensen et al. 1996).

Additionally, the invasive nature of *Salmonella* inhibits contact and transfer of resistance from non-invasive resistant organisms such as *E. coli and E. faecalis* (McClane 1996). This characteristic of *Salmonella* also protects the organism from exposure to the antibiotic. Apramycin is poorly absorbed across the intestinal epithelium due to its low degree of lipid solubility (Mortensen et al. 1996). Limited exposure, in turn, reduces the potential for resistance development.

Resistance to oxytetracycline was expected to develop in the oxytetracycline group around day 28, based on findings from previous research; however, as a similar trend appeared to be developing in *Salmonella,* detection of that organism subsided (Ebner et al. 2000). MICs for poor sanitation were initially significantly higher at day two relative to intermingling, cold stress, and overcrowding treatments but gradually declined to levels comparable to other groups by the end of *Salmonella* detection. It is unlikely that effects of apramycin administration or stress were influential in resistance development as neither antibiotic nor stressor treatment had yet been applied. Although these results are perplexing, levels of resistance for all treatments remained considerably low throughout the study. Throughout the study MICs within the overcrowding treatment were consistent with other rooms, excluding day 14, which yielded a substantial increase in resistance levels. All resistant bacteria were isolated from 3 of the 10 pigs in the treatment. Several factors may have contributed to the

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development of resistance among these isolates. It is possible that either these pigs were stressed more than others for that day, therefore eliciting expression of resistance to oxytetracycline, or interaction with other resistant organisms (*E. coli, E. faecalis*) may have conferred R-factors to those *Salmonella* isolates. It should be noted that no such increase was noted with any other antibiotic for this day.

Although all treatments generally demonstrated a decline in sulfamethazine resistance by day 14, followed by a return to initial levels by day 64, isolates exhibited high MICs throughout the study. An exception to this was the crowding room, which was characterized by a gradual increase in resistance throughout the course of the study. The frequent occurrence of sulfamethazine resistance in production environments as a result of its widespread usage was reflected in the high incidence of resistance detection within all treatments.

E. coli

E. coli exhibited the most pronounced effects of stress on apramycin resistance development. The emergence of apramycin resistant organisms following subtherapeutic administration paralleled findings from previous research investigating the impact of antibiotic supplementation on resistance development (Langlois et al. 1983, Mathew et al. 1998). Resistance, although detected in all treatments receiving apramycin, was prolonged in stressed treatments, particularly cold stress, crowding, and intervention with a second antibiotic (oxytetracycline), relative to the control receiving apramycin. This

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tendency suggests that the application of stress, coupled with subtherapeutic antibiotic administration may have stimulatory effects on resistance development.

The prominence of resistance in the cold stressed group may be reflective of younger pigs' inherent tendency to be less tolerant of colder temperatures than mature pigs, which may, in turn, result in higher levels of stress. Heat stress appeared to be less influential, as high MIC levels were prolonged relative to either of the controls, but less intense and for a shorter time compared to that of the cold stressed pigs.

While transfer of resistance from pigs fed apramycin to pigs with no exposure to the antibiotic in the intermingling treatment cannot be directly associated, resistant isolates were recovered from the additional pigs during the same period in which shedding of resistant *E. coli* from the antibiotic treated pigs was detected. However, resistance within the non-antibiotic pigs was substantially lower, yielding only one resistant isolate at peak resistance and five for the following two days combined. Based on results from this study, it appears that increasing the pig numbers may enhance the effects of stress on resistance development. Increasing the number of pigs in a pen has also been associated with decreased levels of B cell proliferation, which has been associated with an increase in stress (Pohl et al. 1999).

Increased detection of resistant organisms within the oxytetracycline group suggested intervention with a second antibiotic may also promote resistance. Although apramycin and oxytetracycline belong to two different classes of antibiotics (aminoglycosides and tetracyclines, respectively), their

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general mechanisms of action are similar (targeting the 30s ribosomal unit in protein production). It has been suggested that the "predominant" mechanism of resistance to apramycin is due to aminoglycoside modifying enzymes, but may also be a result of decreased transport across cell membranes or modification of the ribosome, itself (Mortensen et al. 1996). Based on these results, the possibility of a connection between resistance development for both of these antibiotics should not be eliminated.

While the poor sanitation treatment demonstrated higher MIC levels for a longer duration than either of the controls, isolates from these pigs maintained resistance for a shorter period than expected. This may be attributed to the elevation of the pens. Although increases in fly numbers and the presence of ammonia added to the stress of the pigs, a reduction of potential for constant exposure to fecal matter may have limited the opportunity for re-infection by resistant organisms. In future studies this factor should be taken into consideration.

It may be possible that *E. coli* elicited a stronger response in resistance to apramycin than *Salmonella* because it is a resident organism of the gut. This gives *E. coli* an advantage over *Salmonella* in that, as an established organism, there are higher numbers and less chance of elimination by the immune system.

Acute stress such as transportation has been documented to increase gut motility thereby, intensifying excretion of intestinal contents (Barone et al.1990). Previous research has also associated this increase with the increased shedding of resistant organisms (Langlois et al. 1999). Slight elevations in MICs 24 h

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post-transport were noted, however, no statistical difference was detected. A previous study detected increase resistance within 1h post-transport and to a lesser degree 24h post-transport (Langlois et al. 1999). It is possible that we missed the optimal time for shedding of resistant organisms by waiting 24h to sample. For future studies, a sampling at 1 and 24 hours post-transport could offer a better insight as to the effects of transportation on resistance development.

The high levels of sensitivity to ceftiofur for *E. coli* within all treatments throughout this study reflect of the strict limitations on use of ceftiofur in the livestock industry and the effective nature of the drug against gram-negative bacteria (Prescott 1993).

In contrast, the widespread use of tetracyclines has contributed to the rise in resistance among a growing number of organisms. *E. coli* isolates generally reflected this trend in the expression of resistance continuously throughout this study. Research has found that detection of resistance to oxytetracycline no longer is primarily associated with antibiotic administration (Guinee 1971). Furthermore, those organisms that do develop resistance from antibiotic administration tend to express those genes for months and even years (Guinee 1971, Langlois 1983).

The extensive agricultural use of sulfamethazine in the past has also contributed to an increased number of resistant organisms. Resistance in this experiment was detected for all treatments after the first sampling ranging from 13-60% of all *E. coli* isolates over the course of the study. High fluctuating levels

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of resistance within our control without antibiotic exposure, suggests that expression of resistance, similar to the situation of oxytetracycline, is no longer primarily dependent on antibiotic exposure. However, in the control administered apramycin, there was a reduction in resistant organisms following removal of the drug; therefore, influence of antibiotic therapy may be a possible factor in resistance development. The effects of stress in combination with antibiotic administration, however, were difficult to determine. It appears that resistance remained relatively constant or gradually increased for all treatments throughout the study. Pigs administered oxytetracycline were among those exhibiting increased levels of resistance to sulfamethazine. Although this increase may not be directly related to the antibiotic treatment, this possibility should be considered in future experiments.

E. Faecalis

Failure to isolate *E. faecalis* throughout the study may have been associated with the colonization of the challenge organism. As detection levels of *S.* Typhimurium waned after day 5, fecal *E. faecalis* concentrations returned to normal levels. Antagonism between indigenous colonic microflora and pathogens often results in a reduction of the pathogenic organism (Ushijima et al. 1991). However, the high dose of *Salmonella* in this study may have had an inhibitory effect on *E. faecalis* upon infection. A combined effect of immune clearance and re-establishment of gut microflora may have resulted in the simultaneous reduction of *Salmonella* and increase in *E. faecalis*. Although

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reliable resistance trends were not detectable in this study an evaluation of the general characteristics of resistance were made. *E. faecalis* generally demonstrated higher MICs than either *E. coli* or *Salmonella* for all antibiotics. Intrinsic resistance has been associated with *E. faecalis* against low levels of aminoglycosides and various cephalasporins, which would explain the high percentage of isolates resistant to ceftiofur across all treatments and days (Knudtson 1993).

Alternatively, *E. faecalis* has also exhibited acquired resistance to oxytetracycline and high levels of aminoglycosides (HLAR) (Knudtson 1993). Knutdson et al. (1993) reported as high as 88% of pork isolates from slaughtering plants to be resistant to oxytetracycline, whereas in this study all isolates exhibited resistance to oxytetracycline. Previous research has indicated that increased resistance to oxytetracycline paralleled administration of antibiotics (Kaukas 1988). However, high percentages of resistant isolates in the control without exposure to antibiotics indicated that resistance expression may have been attributed to the high frequency of resistance genes within the population rather than the influence of antibiotic therapy.

The significance of the establishment of high and low levels of resistance is pertinent to synergistic treatment of *E. faecalis* infections in humans with aminoglycosides and cell wall targeting agents (Rice 1995). Isolates exhibiting HLAR are typically less responsive to the synergistic effects of this treatment. The moderate to high levels of apramycin resistance detected among all isolates in this study substantiate the concerns for treatment.

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The high incidence of multiple resistance exhibited by *E. faecalis* in comparison to *E. coli* and *S.* Typhimurium further complicates treatment concerns. These multiple resistance genes may then be transferred to pathogenic strains through the same mechanisms as described earlier.

In conclusion, the exact mechanisms involved in the development of antibiotic resistance in response to stressors are not fully understood. However, changes within the host's physiological responses to stress (immune response and gut motility) may play a significant role in determining resistance development. As the host is subjected to less favorable conditions, the immune system becomes compromised resulting in bacterial proliferation, possibly by pathogenic strains. Transfer of resistance determinants from indigenous microflora to these pathogenic organisms has been well documented and may ultimately result in treatment failure (Chalsus-Dancla 1986). Further complicating the situation, may be the successful elimination of susceptible isolates through antibiotic therapy, whereas resistant organisms thrive due to lack of competition.

Implications

The increased emphasis on rapid growth and disease prevention in animal husbandry has amplified the use of sub-therapeutic levels of antibiotics. Research linking this type of antibiotic administration to the emergence of resistant bacteria has elicited a growing concern among consumers of agricultural products as well as human medical experts. The objective of this

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study was to evaluate the impact of various environmental and management conditions on bacterial resistance in swine production. Results indicate that the development of *E. coli* resistance to apramycin is significantly increased upon exposure to various stressors. This resistance may be maintained up to 7 weeks following withdrawal of antibiotics from feed. Although S. Typhimurium did not develop resistance in this study, the potential threat of resistance transfer from commensal microflora to food borne pathogens remains a concern for health care specialists. The findings in this study indicate that antibiotic resistance may be controlled by the implementation of proper management strategies coupled with the sensible application of antibiotics. In doing so, producers could simultaneously maximize production efficiency and generate a safer product for consumers.

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APPENDIX

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Table 1: Treatment Groups

Treatment	Description	Number of pigs
Control without Apr (Control-1)	Optimal production conditions	6
Control with Apr (Control-2)	Optimal conditions plus fed apramycin	6
Cold Stress/Apr	6.5^{o} C reduction in recommended temperature plus apramycin treatment	6
Heat Stress/Apr	6.5^{o} C increase in recommended temperature plus apramycin treatment	6
Oxytetracycline/Apr	Fed off label subtherapeutic levels of oxytetracycline (100g/ton TM-50 Type A Pfizer Inc.,) plus apramycin treatment	6
Poor Sanitation/Apr	Monthly accumulation of manure plus apramycin treatment	6
Overcrowding/Apr	30% reduction in floor space plus apramycin treatment	10
Intermingling/Apr	Apramycin treatment plus contact with additional challenged pigs not exposed to apramycin	12

Table 2: Diet Composition (Phase 1)[†]

Feed Ingredient	**Percent of Diet (%)**		
	Control	**Apramycin**	**Apramycin +Oxytetracycline**
Corn	57.62	57.05	57.00
Soybean Meal (48% CP)	25.46	25.21	25.18
Premix[a]	2.62	2.59	2.59
Leanpak[b]	1.73	1.71	1.71
Liquid Energy[c]	2.10	2.07	2.07
Fat Mix[d]	10.483	10.37	10.36
Apramycin (7.5g/lb)	-	0.99	.99
Oxytetracycline (50g/lb)	-	-	.10
Total	100	100	100

[†] Based on a 20% protein, 1.01% lysine
[a] Co-op Swine Base Mix "50" (Tennessee Farmers Cooperative, LaVergne, TN)
[b] Co-op L>E>A>N PAK (Tennessee Farmers Cooperative, LaVergne, TN)
[c] Liquid energy (Tennessee Farmers Cooperative, LaVergne, TN)
[d] Co-op FAT MIX "30" (Tennessee Farmers Cooperative, LaVergne, TN)

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Table 3: Diet Composition (Phase 2)†

Feed Ingredient	Percent of Diet (%)		
	Control	Apramycin	Apramycin +Oxytetracycline
Corn (7.9% CP)	72.5	72.5	72.4
Soybean Meal (48% CP)	15.0	15.0	15.0
Premix[a]	2.5	2.5	2.5
Leanpak[b]	10	10	10.0
Liquid Energy[c]	-	-	-
Fat Mix[d]	-	-	-
Apramycin (7.5g/lb)	-	-	-
Oxytetracycline (50g/lb)*	-	-	.10
Total	100	100	100

*Oxytetracycline (50g/lb) was added (0.1% of diet) to Oxytetracycline treatment in all phase diets, whereas all other treatments received the same diet after withdrawal of apramycin.
[a] Co-op Swine Base Mix "50" (Tennessee Farmers Cooperative, LaVergne, TN)
[b]Co-op L>E>A>N PAK (Tennessee Farmers Cooperative, LaVergne, TN)
† Based on a 18% protein, 0.83% lysine as recommended by NRC requirements (1998).

Table 4: Antibiotic Dilutions and Breakpoints (NCCLS 1996)

Antibiotic	Dilution Range (ug/mL)		Breakpoint (ug/mL)	
Apramycin Sulfate	2-128	8-512 *	≥32	≥512 *
Ceftiofur Sodium	.5-32		≥8	
Oxytetracycline	2-128		≥16	
Sulfamethazine	8-512		≥256	

* E. faecalis high-level resistance testing only

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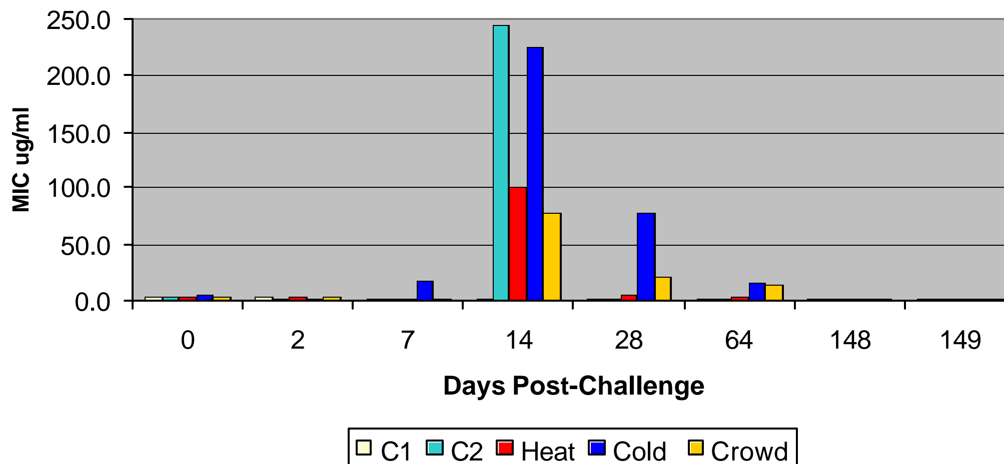

Figure 1. Sensitivity to apramycin in *E. coli* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total number of isolates = 1175
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatment effects (P < .05)

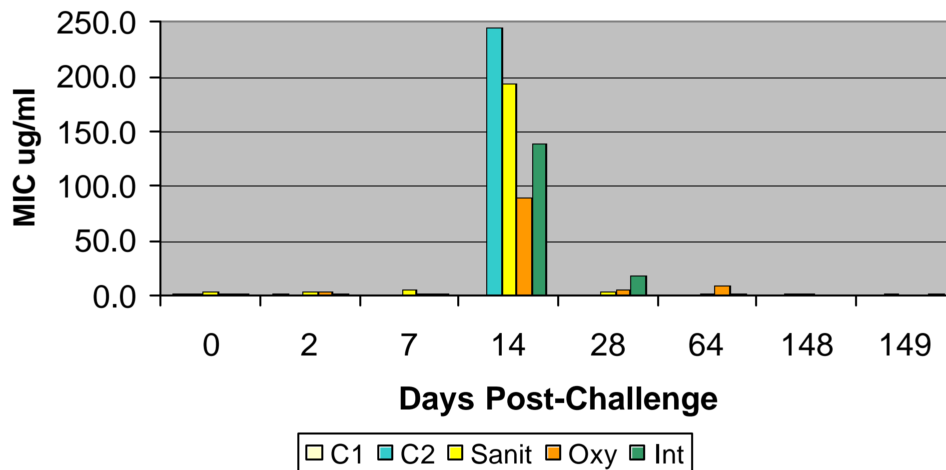

Figure 2. Sensitivity to apramycin in *E. coli* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total number of isolates = 1175
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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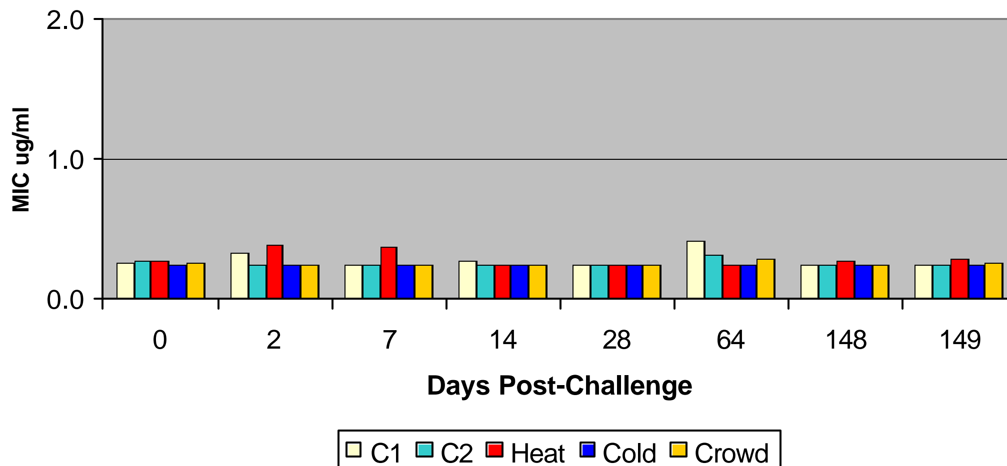

Figure 3. Sensitivity to ceftiofur in *E. coli* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1178
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments $P < .05$
Treatments effects ($P < .05$)

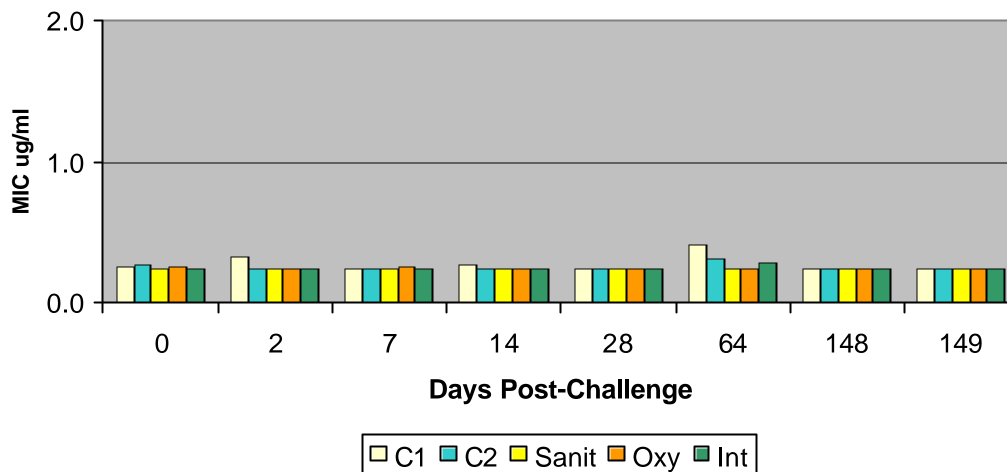

Figure 4. Sensitivity to ceftiofur in *E. coli* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1178
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments $P < .05$
Treatments effects ($P < .05$)

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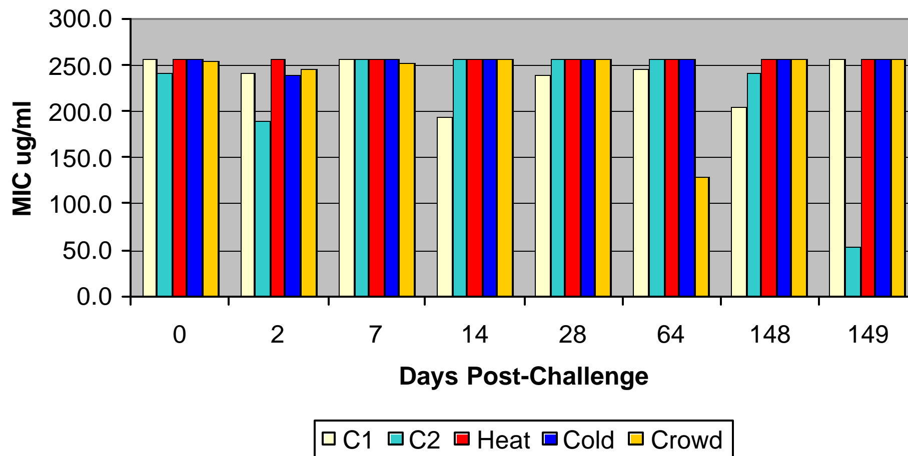

Figure 5. Sensitivity to oxytetracycline in *E. coli* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1176
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatments effects (P < .05)

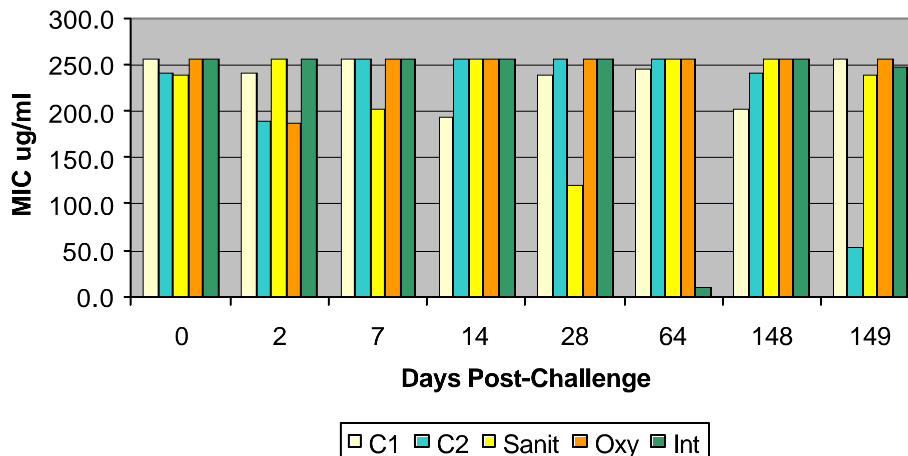

Figure 6. Sensitivity to oxytetracycline in *E. coli* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1176
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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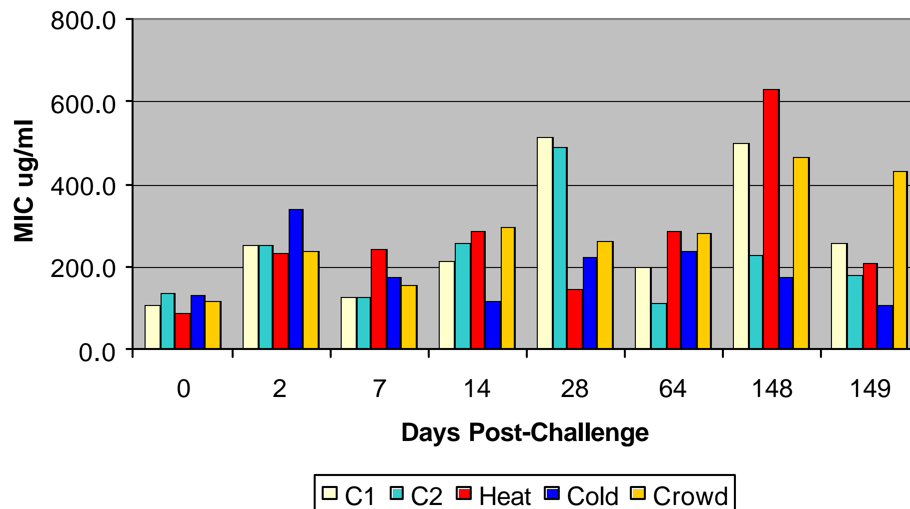

Figure 7. Sensitivity to sulfamethazine in *E. coli* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1173
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P< .0001
Treatments effects (P < .05)

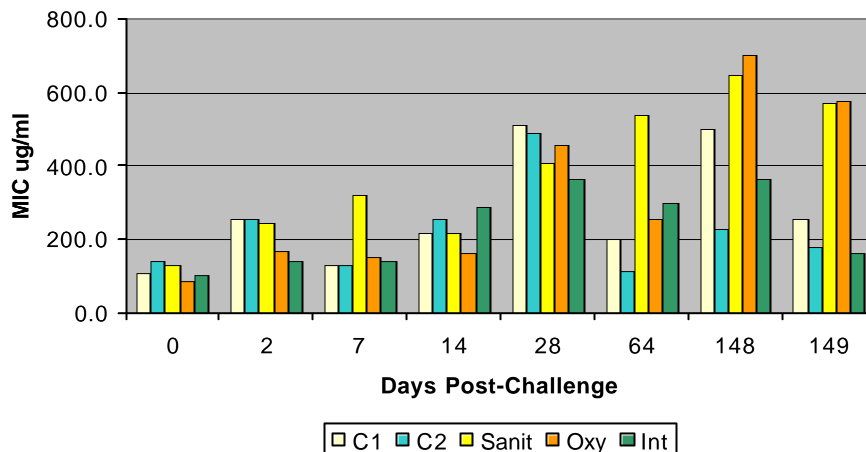

Figure 8. Sensitivity to sulfamethazine in *E. coli* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 1173
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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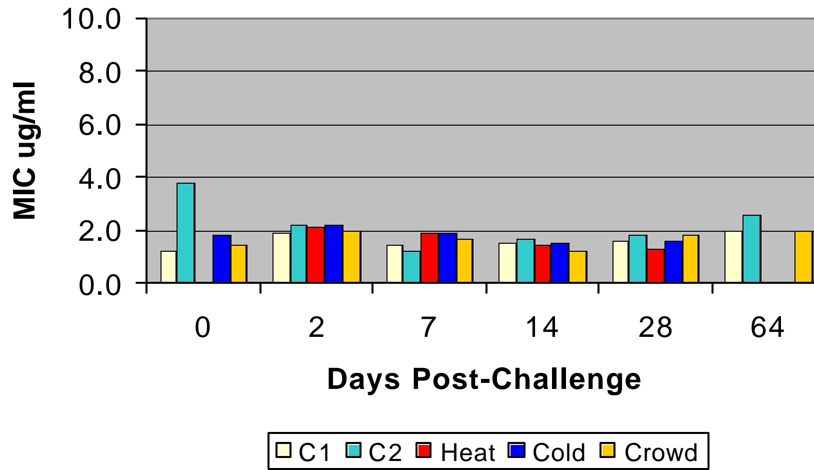

Figure 9. Sensitivity to apramycin in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 727
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .05
Treatments effects (P > .05)

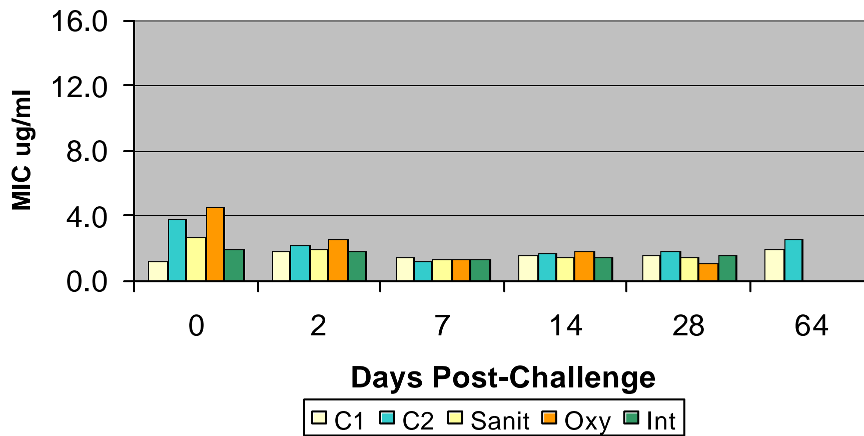

Figure 10. Sensitivity to apramycin in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter
Total Number of isolates = 727
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P < .05
Treatments effects (P > .05)

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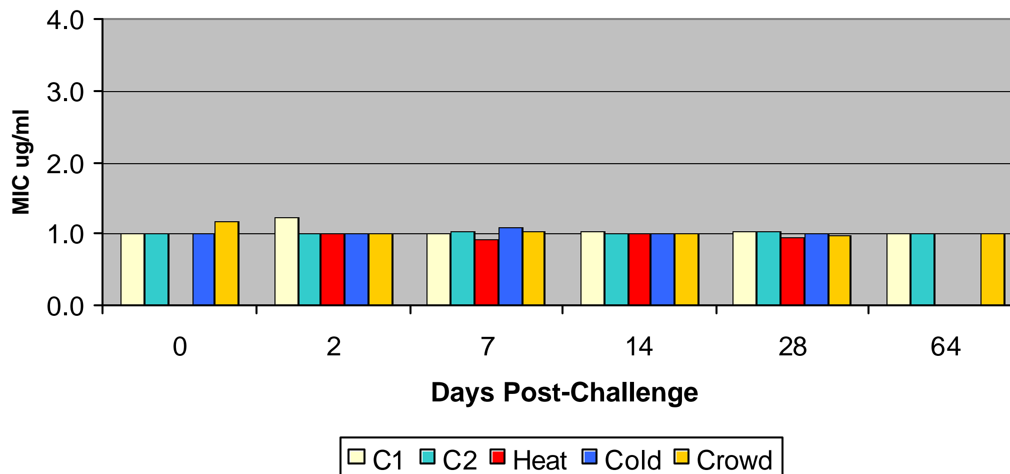

Figure 11. Sensitivity to ceftiofur in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 729
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatments effects (P > .05)

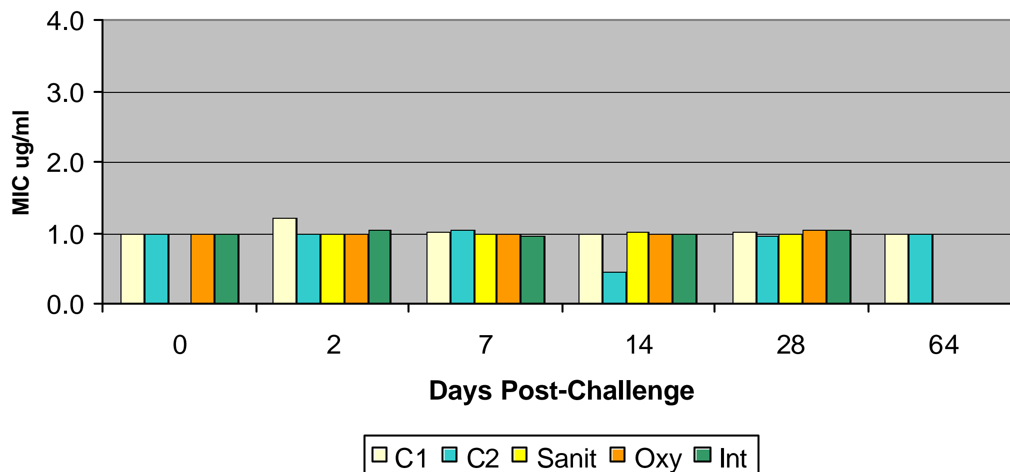

Figure 12. Sensitivity to ceftiofur in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 729
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P >.05)

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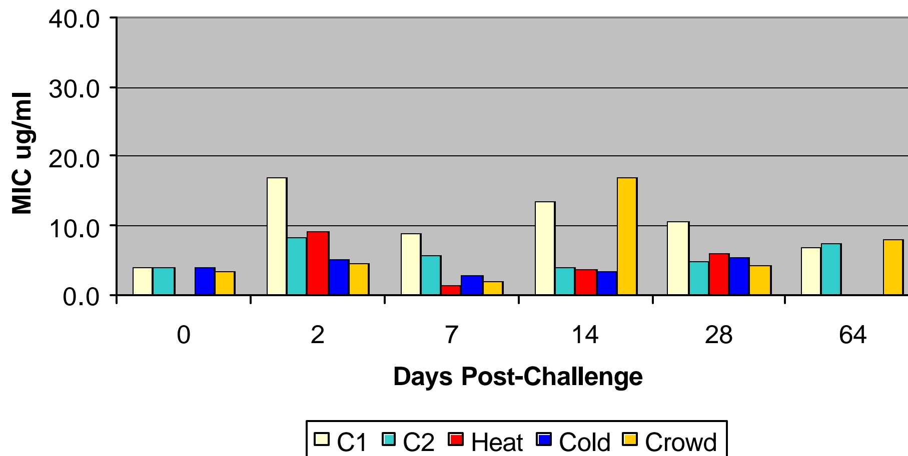

Figure 13. Sensitivity to oxytetracycline in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 630
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P <.05
Treatments effects (P < .05)

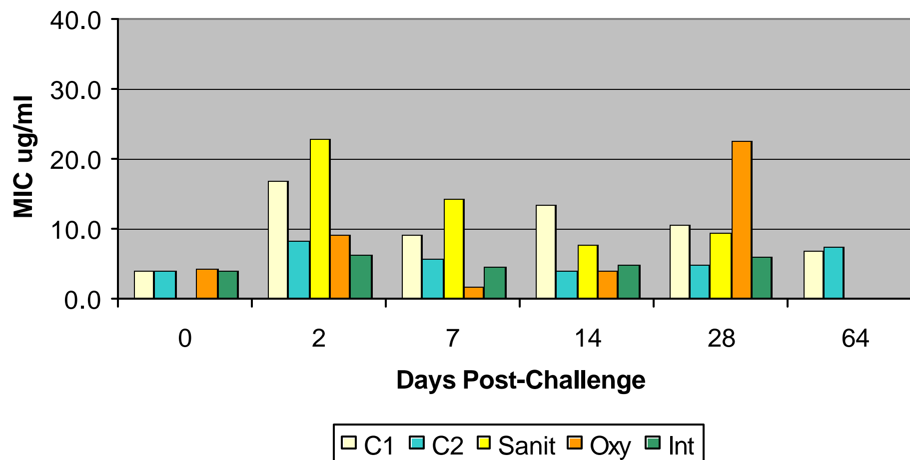

Figure 14. Sensitivity to oxytetracycline in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 630
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy = Oxytetracycline, Int = Intermingling
Time differences within treatments P <.05
Treatments effects (P < .05)

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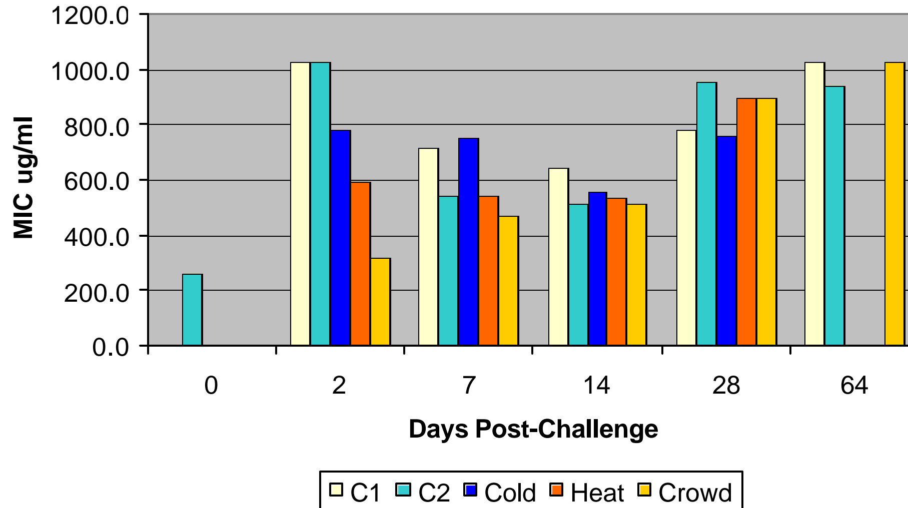

Figure 15. Sensitivity to sulfamethazine in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 729
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatments effects (P < .05)

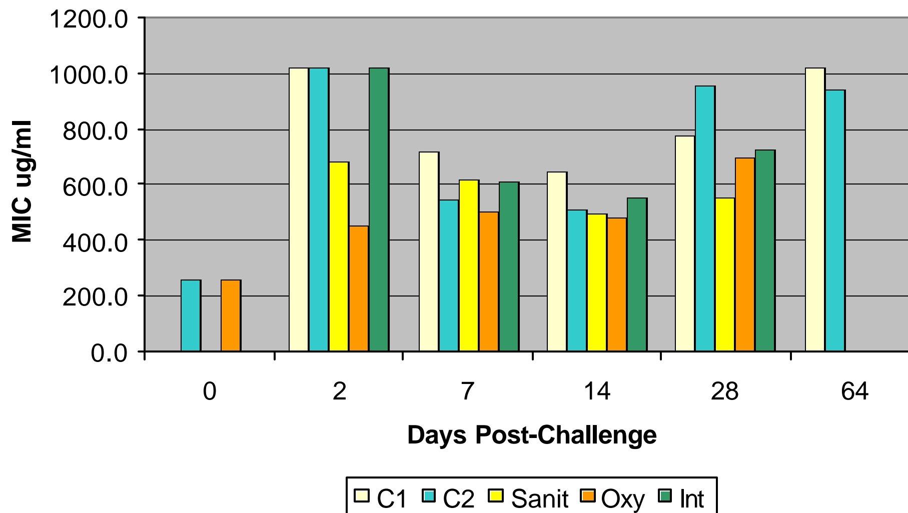

Figure 16. Sensitivity to sulfamethazine in *S.* Typhimurium isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 729
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy= Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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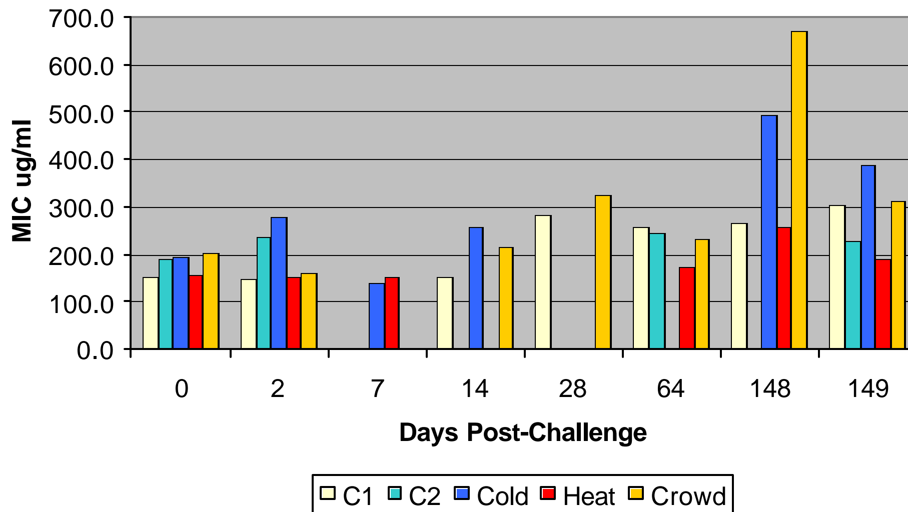

Figure 17. Sensitivity to apramycin in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 844
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatments effects (P < .05)

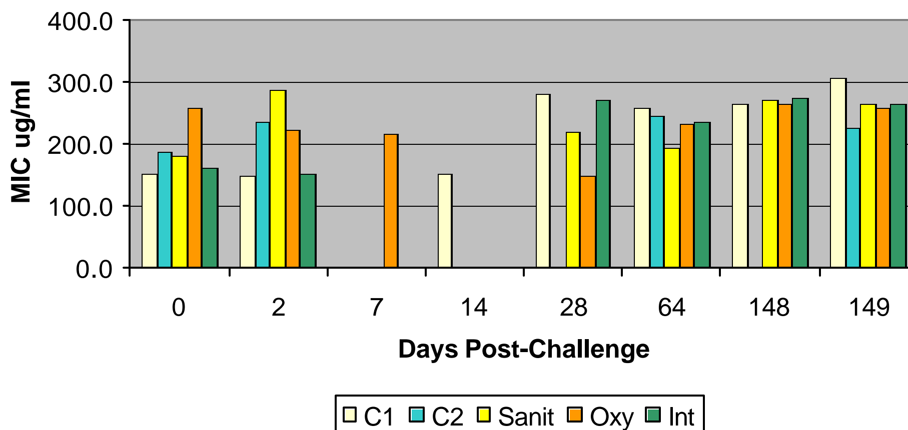

Figure 18. Sensitivity to apramycin in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 844
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy= Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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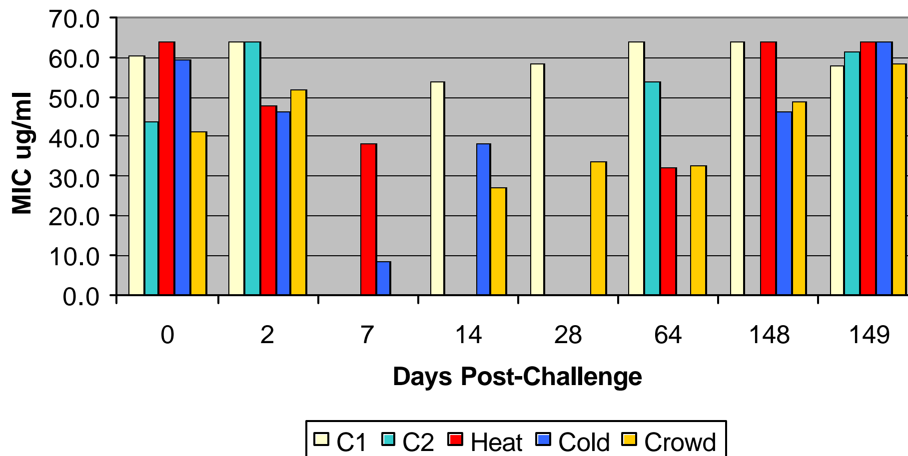

Figure 19. Sensitivity to ceftiofur in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 844
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P < .0001
Treatments effects (P < .05)

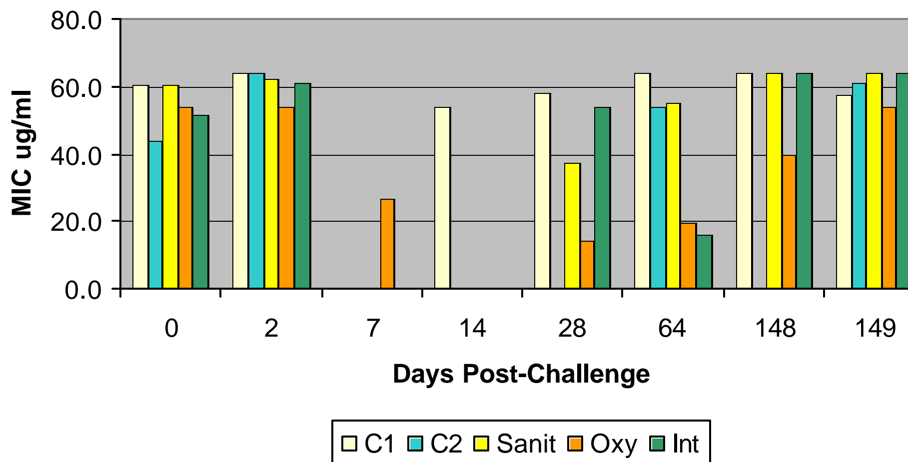

Figure 20. Sensitivity to ceftiofur in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 844
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy= Oxytetracycline, Int = Intermingling
Time differences within treatments P < .0001
Treatments effects (P < .05)

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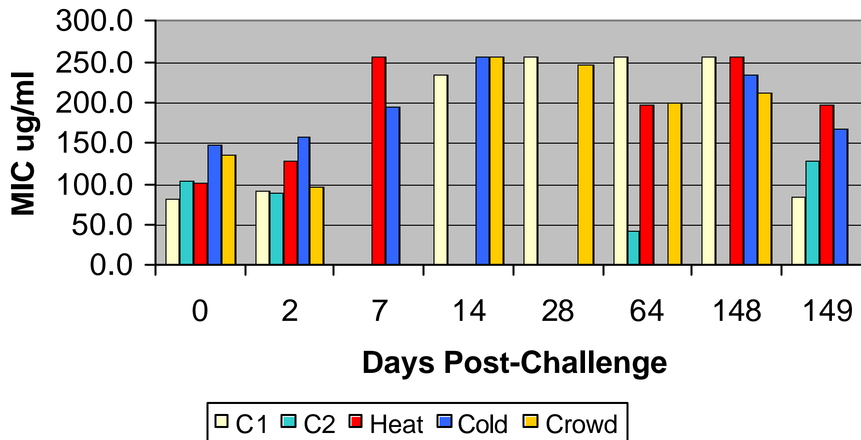

Figure 21. Sensitivity to oxytetracycline in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 838
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P > .05
Treatments effects (P < .05)

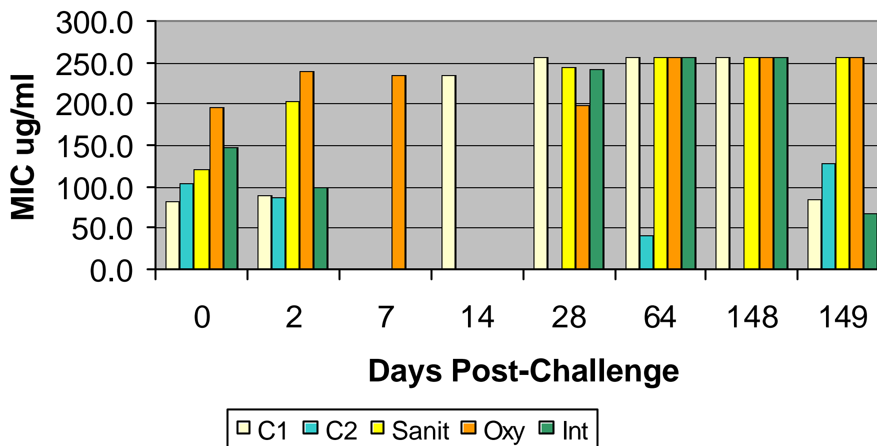

Figure 22. Sensitivity to oxytetracycline in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 838
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy= Oxytetracycline, Int = Intermingling
Time differences within treatments P > .05
Treatments effects (P < .05)

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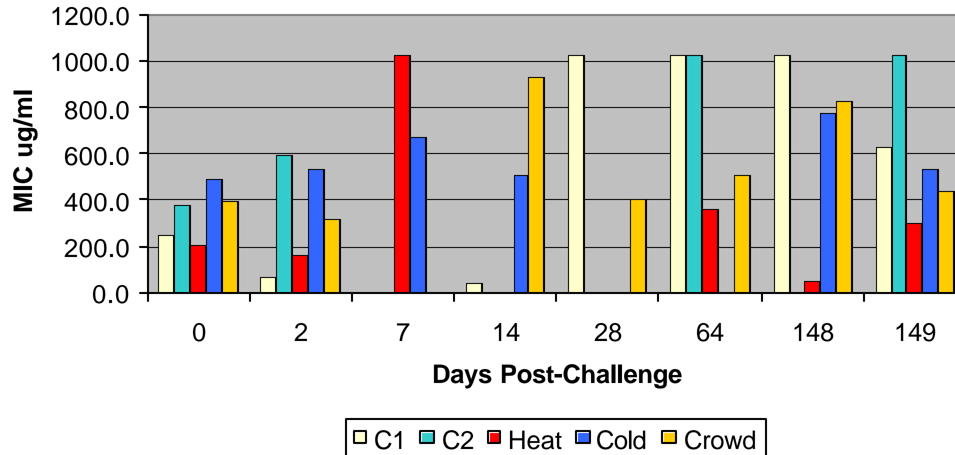

Figure 23. Sensitivity to sulfamethazine in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, heat, cold, and crowding treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter.
Total Number of isolates = 844
C1 = control without apramycin, C2 = control with apramycin, Heat = Heat Stress, Cold = Cold Stress, Crowd = Overcrowding
Time differences within treatments P > .05
Treatments effects (P < .05)

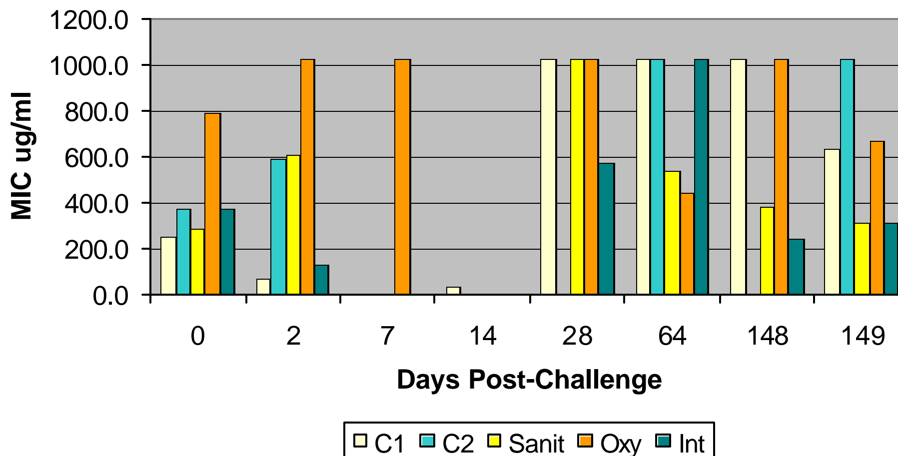

Figure 24. Sensitivity to sulfamethazine in *E. faecalis* isolated from pigs within control without apramycin, control with apramycin, poor sanitation, oxytetracycline, and intermingling treatments over time
Data are presented as Least Squares Means of minimum inhibitory concentrations (MIC) in micrograms per milliliter
Total Number of isolates = 844
C1 = Control without apramycin, C2 = Control with apramycin, Sanit = Poor Sanitation, Oxy= Oxytetracycline, Int = Intermingling
Time differences within treatments P > .05
Treatments effects (P < .05)

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VITA

Patricia Cullen was born and raised in Nashville, TN before attending Clemson University in 1995. The following year she transferred to the University of Tennessee, Knoxville, where she completed her undergraduate studies in Animal Science. Upon completion of her Bachelors degree, she continued her education at Tennessee working as a graduate research assistant in the Department of Animal Sciences. During her time in the Masters program, she served as Vice-President for the Animal Science Graduate Student Association. She is also a member of the American Society for Microbiology as well as the American Society of Animal Science. Upon completion of her Masters degree, she hopes to pursue a career in microbiology and research.

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