RESEARCH REPORT
PORK SAFETY

Title: Use of cationic peptides as feed additives to improve innate immunity and reduce gut colonization with *Salmonella* and *Campylobacter* in weaned pigs **- NPB #09-099**

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Industry Summary
The objectives of this study were to determine if the addition of a cationic peptide to the feed formulation of weaned pigs decreases *Salmonella* and *Campylobacter* colonization of the pig's gut and to investigate the effects of using cationic peptides as feed additives in weaned pig rations on the innate immune response of weaned pigs. Pigs were fed a feed ration containing 24 ppm of the BT TAMU cationic peptide for the duration of each part of the studies. In the *Salmonella* studies, pigs in both control and peptide groups were given *Salmonella* orally. Daily rectal swabs were taken and pigs were euthanized and cultured for the presence of *Salmonella* on day 7 after infection. Pigs fed the peptide had reduced fecal shedding of *Salmonella* and reduced *Salmonella* in the tissues compared to control pigs that did not receive the peptide. In addition, pigs fed the peptide had higher daily weight gains than did pigs that were not fed the peptide. No significant reductions of *Campylobacter* were found in pigs fed the peptide with preexisting *Campylobacter* infections. In innate immunity studies, one mechanism swine leukocytes use to kill microbes, called an oxidative burst, was found to be higher in pigs fed the peptide versus pigs that did not receive the peptide on all days it was measured. Leukocyte degranulation, another mechanism leukocytes use to kill microbes, was not found to be different between control and peptide-fed pigs. In summary, the addition of the BT TAMU peptide to weaned pig feed resulted in reduced *Salmonella* shedding in feces and reductions in tissue colonization. The peptide also stimulated the immune system of pigs fed the peptide, as indicated by an increased oxidative burst response in these pigs. Although no effects against *Campylobacter* were observed, this may have been due to the fact that all pigs used in the studies had preexisting *Campylobacter* infections. If *Campylobacter*-free pigs were available, the results may have differed. These studies indicate the BT TAMU peptide may be an effective alternative, immunologically-based strategy to the use of antibiotics to reduce *Salmonella* in swine.

Keywords
Feed additive, cationic peptide, *Salmonella*, immune, *Campylobacter*, preharvest

Scientific Abstract
A novel gram-positive bacterium *B. texasporus* (ATCC PTA-5854) previously isolated produces BT/TAMUS 2032 (BT), a group of related cation amphipathic peptides of 13 amino acid residues. The BT peptide has been previously shown to reduce *Salmonella* and prime the innate immune system in chickens. In the present study,

BT was fed to weaned pigs at 12 and 24 ppm as part of a balanced ration. In *Salmonella* studies, piglets were challenged with *Salmonella typhimurium* (ST) and monitored for shedding and weight gain over a 7 day period. Pigs were then humanely sacrificed and intestinal and organ samples were cultured for the presence of ST. Pigs fed the BT peptide had significant reductions in fecal shedding and in tissue invasion of ST as compared to control pigs. Average daily weight gain was also found to be higher in pigs fed BT compared to control pigs. The BT peptide did not significantly reduce the carriage of *Campylobacter* in the swine gut when fed against an preexisting infection. On days 3, 5, and 7, leukocytes from pigs fed the BT peptide had significantly higher production of an oxidative burst when compared to control levels ($P > 0.05$). Leukocyte degranulation was not found to differ between groups. The data suggest that the BT peptide may be an effective immunomodulator with subsequent effects on the carriage of *Salmonella* in both the swine gut and tissues.

V. Introduction

It is becoming increasingly recognized that the therapy of infectious diseases of swine, particularly food-borne bacterial infections, is facing twin threats. On one hand antibiotic resistance is rising; on the other hand there are relatively few novel compounds under development. The search for broad spectrum therapeutics that do not trigger bacterial resistance has generated interest in human medicine in the natural antimicrobial peptides of the innate host defenses. This peptide immune system is evolutionarily ancient and is found in virtually all eukaryotes from insects to mammals (Hoffman *et al*., 1999; Hancock and Falla, 1996). These peptides play a primary role at the local mucosal and epithelial surfaces, providing early innate effector defenses against infection (Bals, 2000). Antimicrobial peptides (AMPs) are broad-spectrum with potent antimicrobial activity against human and veterinary pathogens that qualify as prototypes of novel and innovative drugs. Therapeutically, the cationic peptides represent the most promising of these potential drugs because of their ability to up-regulate protective innate immune responses while suppressing the potentially harmful inflammatory responses (Finlay and Hancock, 2004; Bowdish *et al.*, 2005). Two families of AMPs that have received the most characterization in the public and veterinary medical fields are the defensins and cathelicidins (Yang et al., 2004; Bals and Wilson, 2003; Brogden *et al.*, 2003).

Mechanistically, what separates the cationic peptides from traditional antibiotics is that many do not act directly on the bacteria ; i.e., affecting or interrupting normal bacterial function, that prevents the emergence of resistant strains of bacteria. Instead, the cationic peptides act directly on cells of the innate immune system (Hancock, 2001; Scott *et al.*, 2007). Specifically, these peptides subtly enhance specific host innate immune responses and increasing infection-resolving immunity while diminishing potentially harmful pro-inflammatory responses (sepsis).

It has long been known that microbes produce a variety of secondary metabolites to compete with other microbes for ecological niches. A novel gram-positive bacterium *B. texasporus* (ATCC PTA-5854) has been isolated that produces BT/TAMUS 2032 (BT), a group of related cation amphipathic peptides of 13 amino acid residues (Wu *et al.*, 2005). *In* vitro, BT displays efficient bactericidal activity against gram-positive bacteria (minimal inhibition concentration [MIC] of 1 ppm), but a reduced efficacy against gram-negative bacteria (MIC > 20 ppm). However, in a recent study BT was found to protect chickens against a natural outbreak of colibacillosis without directly affecting the bacteria since the protective concentration (12 ppm) was below the MIC for *E. coli* (Jiang *et al.*, 2005). More surprisingly, orally delivered BT seems to be completely lacking direct antibacterial activities.

Presently, the swine industry is under immense pressure to identify novel approaches to the control of pathogens in swine that curtail and/or eliminate the use of antibiotics. Immuno-modulation represents a strategy that uses the animal's normal biologic functions to control and eliminate pathogens without the requirement of antibiotics. If the stimulation of the early innate immune response can aid in the reduction in carriage of pathogens in piglets and subsequently throughout production, this strategy would be a beneficial tool for both the reduction of food born and swine pathogens. This would meet the producer's requirement of delivering safe products to the consumer and would aid in reducing the use of antibiotics and subsequent spread of resistance among bacteria. In addition, stimulation of the innate system early in life might allow piglets to be weaned earlier than pigs are weaned presently. Earlier weaning would allow sows to be re-bred earlier and subsequently reduce the time between litters for the individual sow.

Objectives:
1. Determine if the addition of a cationic peptide to the feed formulation of weaned pigs decreases *Salmonella* and *Campylobacter* colonization of the pig's gut.

2. Investigate the effects of using cationic peptides as feed additives in weaned pig rations on the innate immune response of weaned pigs.

Materials and Methods
Objective 1: Does the addition of the cationic peptides to a feed ration for the first week post-weaning affect the susceptibility of newly weaned pigs to intestinal and/or organ colonization by *Salmonella enterica* serovar *Typhimurium* (ST), and *Campylobacter coli* (CI)?
At weaning (17-21 days of age), piglets will be randomly placed in pens with heat lamps for additional warmth in groups of 5 individuals. Piglets will be provided with water and an un-medicated Phase I diet *ad libitum* that meets the National Research Council (1994) guidelines (diet provided by D. Knabe, Texas A&M University). Body weights will be recorded on Days 0, 3, 5, and 7 post-weaning. Utilizing a completely randomized block design, pigs will be placed into 4 experimental groups. Groups 1 and 2 will each contain 5 pigs fed a control balanced unmedicated corn and soybean meal-based diet that will contain 0 or 12, ppm BT, respectively. Groups 3 and 4 will be fed diets that contain 0 or 12 ppm BT, but pigs in these groups will be challenged with ST or CI, respectively (see experimental designs below). (Peptide concentration in the feed was adjusted to 24ppm following 1st set of experiments with Salmonella). Pigs will be fed these diets throughout the experiments. Experiments for each infection will be repeated for two repetitions. All methods and ideologies presented in this proposal have been reviewed through the USDA, ARS internal review procedures and have been accepted, including the SPARC Animal Care and Use Committee.

Peptide Premix. A gram-positive soil bacterium *Brevibacillus texasporus* produces a group of related cationic amphipathic peptides of 13 amino acid residues (BT). C *B. texasporus* cells will be grown in one liter of LB in a 37^{o}C air shaker for three days. The culture will be spun at 3000 rpm for 15 minutes, the supernatant collected, and 500 grams of ammonium sulfate is then added and dissolved. The sample is then spun at 3000 rpm for 15 minutes. The pellets are dissolved in 200 ml of distilled water. The solution is then boiled for 15 minutes and then cooled on ice. The sample is filtered with a 0.2 micron filter. The filtrate is mixed with 0.2 liter of chloroform at room temperature for 20 minutes with constant stirring. The mixture is then separated into two phases through centrifugation at 3000 rpm for 15 minutes. The organic phase is collected and dried in a vacuum evaporator. The BT peptides are then eluted with ethanol and the ethanol solution is sprayed onto cornmeal and dried to produce the BT premix.

***Salmonella enterica* serovar *Typhimurium* (ST)**. An isolate of *S. enterica* serovar *typhimurium* (ST) (National Veterinary Sciences Laboratory [NVSL]), Ames, IA (PT 24) will be used and has been selected for resistance to carbenicillin and novobiocin (CN) and is maintained in TSB or tryptic soy agar at 4^{o}C. Brilliant Green Agar (BGA), a selective culture media for *Salmonella*, will be used to culture the resistant isolate in experimental studies and contained 100 μg/ml carbenicillin and 25 μg/ml novobiocin (i.e., BGA + CN) to inhibit growth of other bacteria. Inoculum for challenge is prepared from 18 to 24 h (TSB + CN cultures maintained at 41^{o}C and diluted in sterile PBS (pH 7.2). A stock solution (1 x 10^{9} cfu/ml) will be prepared for challenge experiments.

At day 3 post-weaning, piglet in challenge groups will be administered, by oral gavage, 10^{7} cfu ST. Five days post-challenge (7 days post-wean), pigs will be euthanized and 1.0 g samples of cecal contents from each pig will be collected aseptically. The cecal samples will be serially diluted and spread-plated on brilliant green agar (BGA) plates. The plates will then be incubated for 24 h at 37C and the number of cfu of ST per gram of cecal contents determined. In addition, tissues (liver, spleen, ileo-cecal lymph nodes, cecum and rectum will be cultured for the presence of ST using established methods ( ). Suspect *Salmonella* colonies will be confirmed by biochemical tests on triple sugar agar and lysine iron agar and further confirmed as ST by using *Salmonella* O antiserum. *Salmonella* colony plate counts are expressed as $\log_{10}$ *Salmonella* per gram of cecal contents.

Campylobacter coli (CI) A laboratory isolate of CI from a weaned pig has been generously supplied by Dr. Robin Anderson (USDA ARS SPARC). CI will be cultured in Bolton broth at 41 ^{o}C for 40 h under microaerophilic environment (5% O_2, 10% CO_2, 85% N_2), centrifuged and diluted to the desired optical density with PBS (pH = 7.2) based on the correlation between cfu and absorbance. At day 3 post-weaning, piglets will be challenged by oral gavage with 10^5 cfu of CI. At 5 d post-challenge (7 days post-weaning), piglets will be euthanized and sections of the gut (ileum, jejunum, cecum, colon, rectum) will be cultured for the presence of CI. In addition, cecal contents will be serially diluted and plated onto campy cefex plates for a 44 h incubation in a microanaerobic environment (5% O_2, 10% CO_2, 85% N_2) at 41 ^{o}C to determine the number of cfu of CI per gram of cecal contents.

Objective 2: Do the cationic peptides induce an innate immune response in weaned pigs?

Leukocytes will be isolated using density gradient separation as previously described (4,5,8). Functional assays will then be performed to assess the capabilities of neutrophils and monocytes isolated from pigs receiving the different treatments. Two methods of microbial killing used by neutrophils will be investigated. Specifically, neutrophil degranulation, the release of bactericidal products from granules inside the neutrophil, and the oxidative burst, the production of bactericidal reactive oxygen species (ROS) will be assayed. The phagocytosis of *Salmonella* by neutrophils and monocytes will also be observed using established methods. Briefly, the assays are described below. In addition, samples from leukocytes will be collected and frozen for future studies on cytokine gene expression.

Functional assays.
Neutrophil/monocyte phagocytosis of *Salmonella* will be assayed as previously described. Neutrophils, 4 x 10^6 cells/ml in RPMI along with *Salmonella enteritidis (SE),* 1 x 10^8 cfu/ml, 100 ST:1 neutrophil, and incubated for 60 min at 39ºC on a rocker. The samples will then be washed with an equal volume of RPMI and centrifuged at 190 *g* for 10 min and the supernatant discarded. The neutrophils will be washed an additional three times with RPMI, and the pellet resuspended in the original volume. Cytospin smears will be prepared from each sample in a Shandon cytospin3 (Shandon Inc., Pittsburg, PA), stained with Hema 3 stain system (Biochemical Sciences, Inc., Swedesboro, NJ), and examined by light microscopy under oil immersion (100 x). Results are reported as the percent neutrophils containing ST and the average number of ST/neutrophil.

Leukocyte degranulation will be detected by quantifying the amount of β-glucuronidase activity in the culture medium following stimulation of neutrophils (8 x 10^6 cells/ml) with or without opsonized *Salmonella* (OPSE) for 60 minutes at 39 ºC on a rotary shaker in a 5% CO_2 incubator. The reaction is stopped by transferring the tubes containing the cells to an ice bath for 5 min. Cells are then centrifuged at 250g for 10 min at 4 ºC. The supernatants are removed and used in the assay. Each supernatant sample (25µl) is added to 8 wells in a non-treated black CoStar flat-bottom ELISA plate and incubated with 50 µl of freshly prepared substrate (10 mM 4-methylumbelliferone-β-glucuronidide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4h at 41 ºC. The reaction is stopped by adding 200 µl of a stop solution (0.05 M glycine and 5mM EDTA; pH 10.4). Liberated 4-methylumbelliferone is measured using an f_{max} fluorescence microplate reader [(355/460 nm) Molecular Devices, Sunnyvale, CA].

Leukocyte oxidative burst will be measured by oxidation of DCFH-DA to fluorescent DCF as described by He et al., (9). Leukocytes are stimulated with phorbol A-myristate 13 acetate (PMA [2.0 µg/ml], Sigma) or RPMI 1640 media for 60 minutes prior to measurement. Cells are placed in 96-well plates and fluorescence is measured using a GENios Plus Fluorescence Microplate Reader [(485/530 nm) Tecan US Inc., Research Triangle Park, NC]. Statistical analysis: Statistical analysis will be performed on data using SigmaStat® statistical software (Jandel Scientific, San Rafael, CA, USA). Differences between the experimental groups will be determined using analysis of variance. $p \leq 0.05$ will be considered to be statistically significant.

All methods and ideologies presented in this proposal have been reviewed through the USDA, ARS internal review procedures and have been accepted, including the SPARC Animal Care and Use Committee.

Results

From the data presented below, it appears pigs fed the peptide had greater average daily gains in weight (ADG) than did pigs fed the control diet, even in the face of a *Salmonella* infection. Some reductions in fecal shedding of ST was observed as shown in the rectal swab data. Pigs fed the peptide had less *Salmonella* in the organs – lymph nodes, liver, spleen – as compared to the control pigs, but did not show reductions in the gut tissues – ileum (except rep 3), cecum, rectum. Colonization data (CFUs) was indeterminate due to low recovery of ST. It appears the peptide has some positive effects on *Salmonella* invasion and colonization in weaned pigs.
Multiple sets of weaned piglets were acquired and all were cultured for the presence of *Campylobacter* prior to beginning the studies. All pigs were found to be previously colonized with *Campylobacter coli*. Due to preexisting infections, the studies were altered to take into account the preexisting conditions. It is believed that the preexisting *Campylobacter* infections may have caused the peptide to be less effective against *Campylobacter* than would be expected. In the *Campylobacter* studies, no significant reductions in *Campylobacter* positive samples from the pig gut were observed in the peptide groups, although some "biological" reductions were noted. In addition, CFU counts of *Campylobacter* in the cecum were found to be statistically the same for the control pigs and the peptide-fed pigs. The *Campylobacter* data is pooled and summarized in one figure for the gut samples.
In the objective 2 studies, leukocytes from pigs fed the peptide had a significantly higher oxidative burst response than did leukocytes from control pigs on days 3, 5, and 7 of being fed the peptide ($P < 0.05$). Leukocyte degranulation was not found to be different between peptide-fed and control pigs (Figure). Phagocytosis was not performed due to low cell recovery.

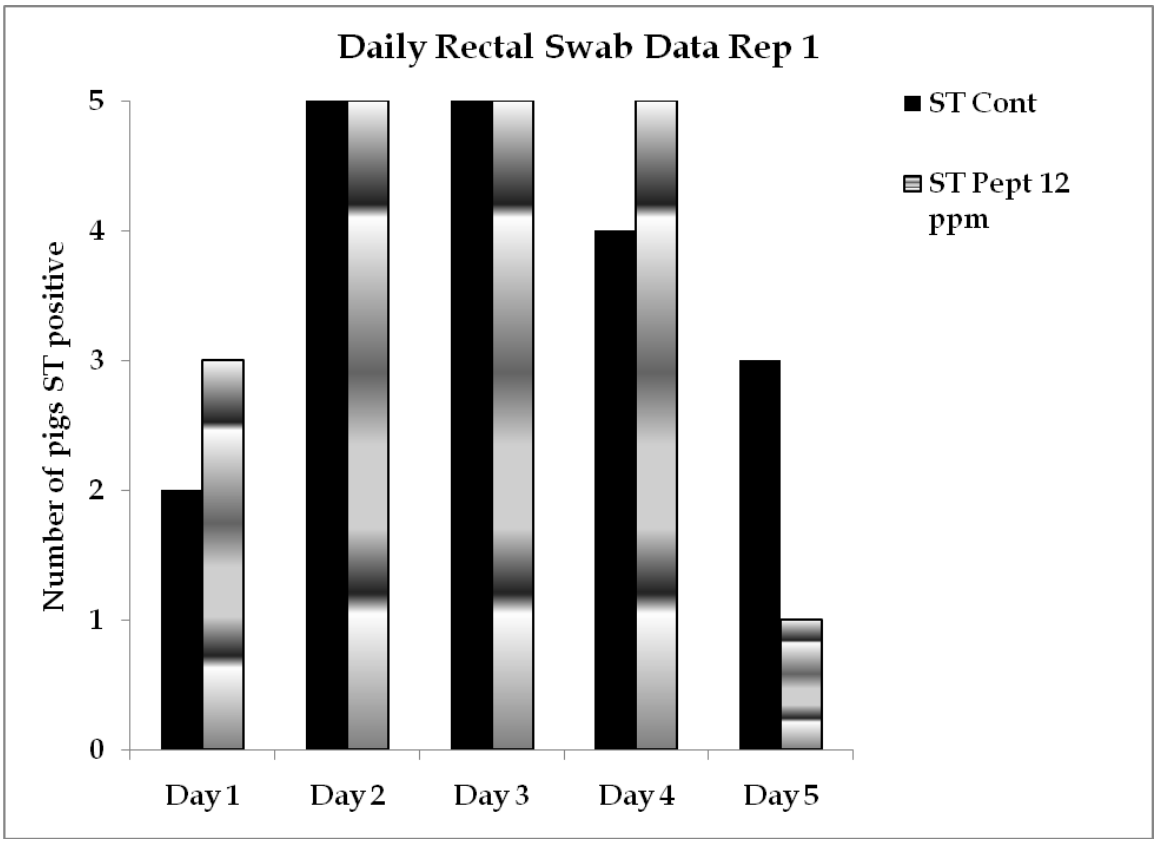
Objective 1:

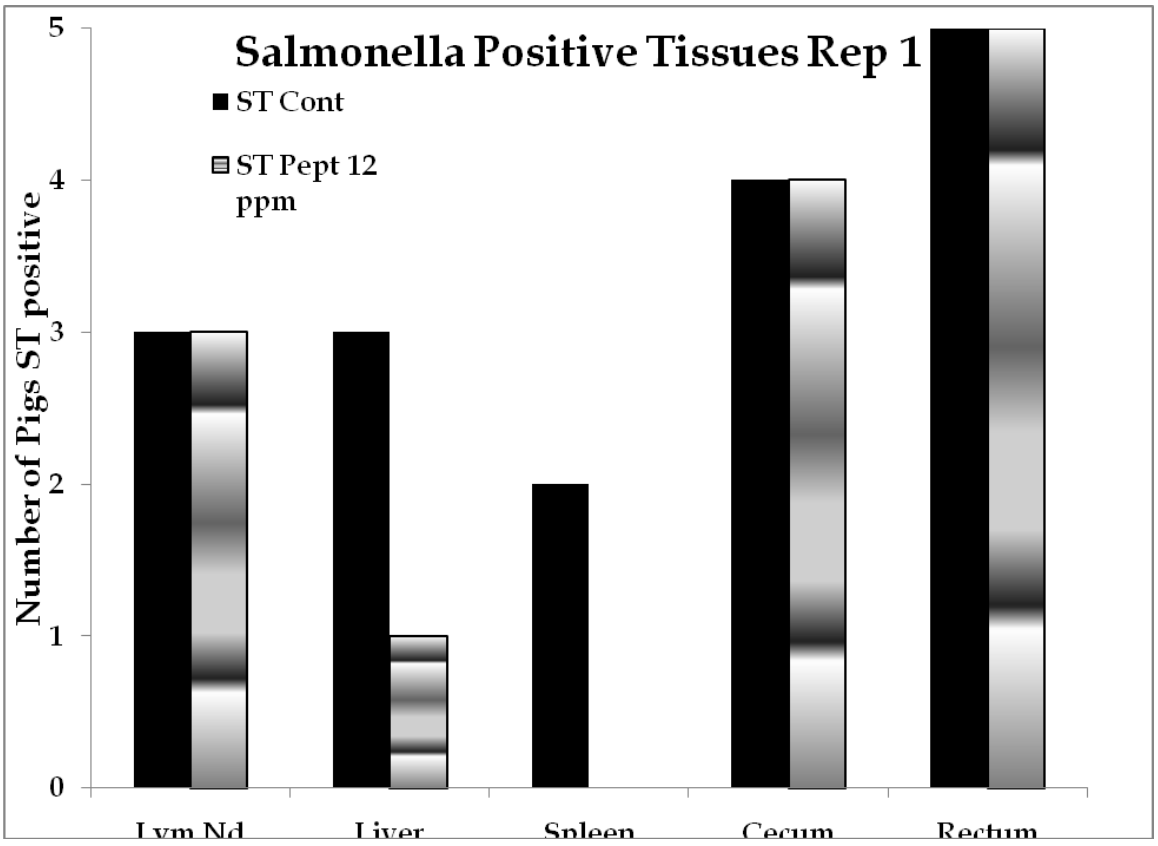
Salmonella Results:

Figure Legends:

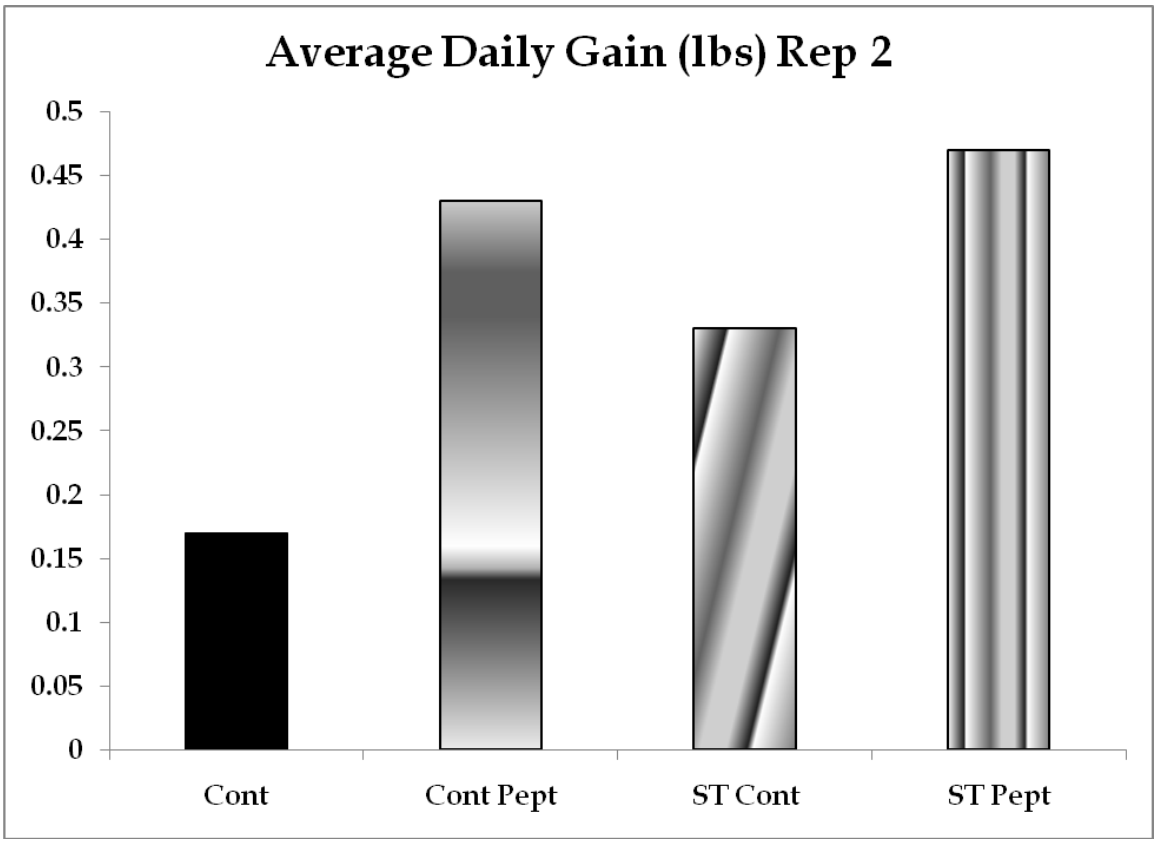
ST – *Salmonella typhimurium*
LN – ileo-cecal lymph node
LIV – liver
SPL – spleen
IL – ileum
Cec – cecum
Rec – rectum
Cont – control feed
Cont Pept – control pigs fed peptide
ST Cont – control feed with ST challenge
ST Pept – peptide feed with ST challenge

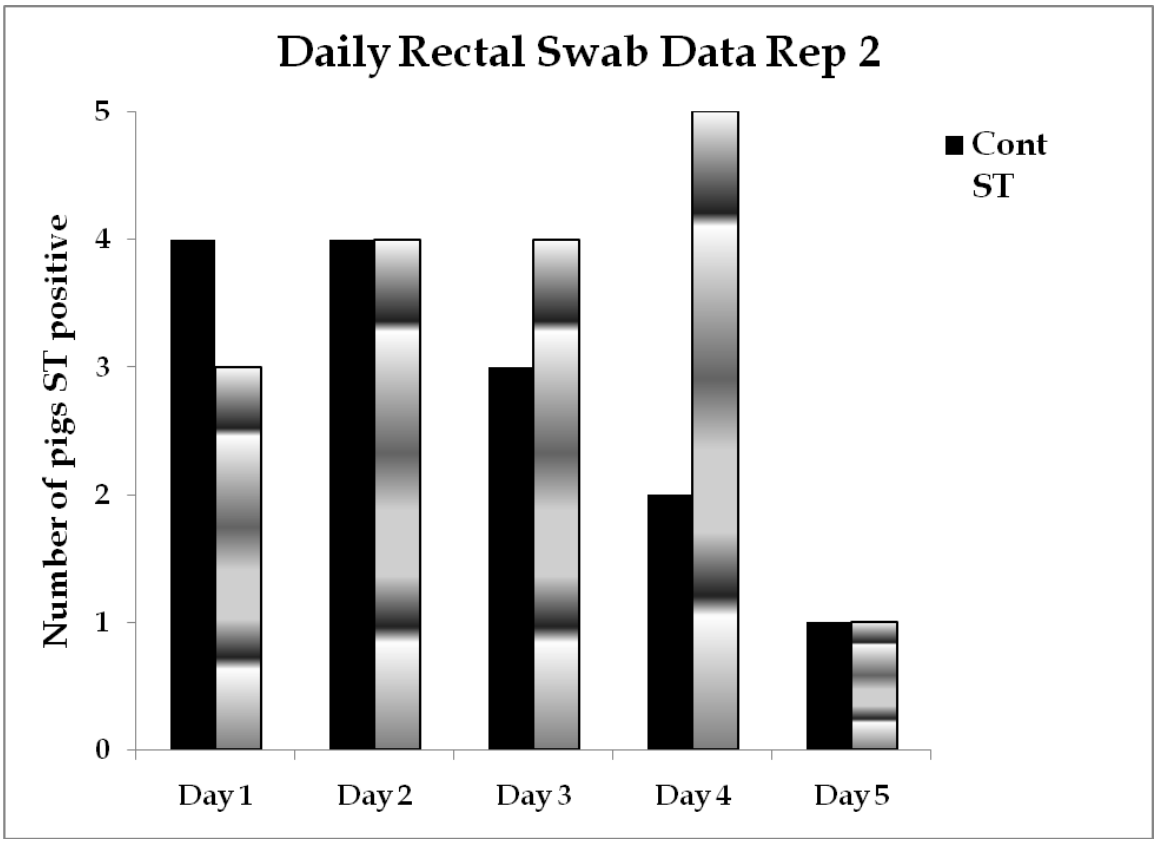
Rep 1 Data: Salmonella

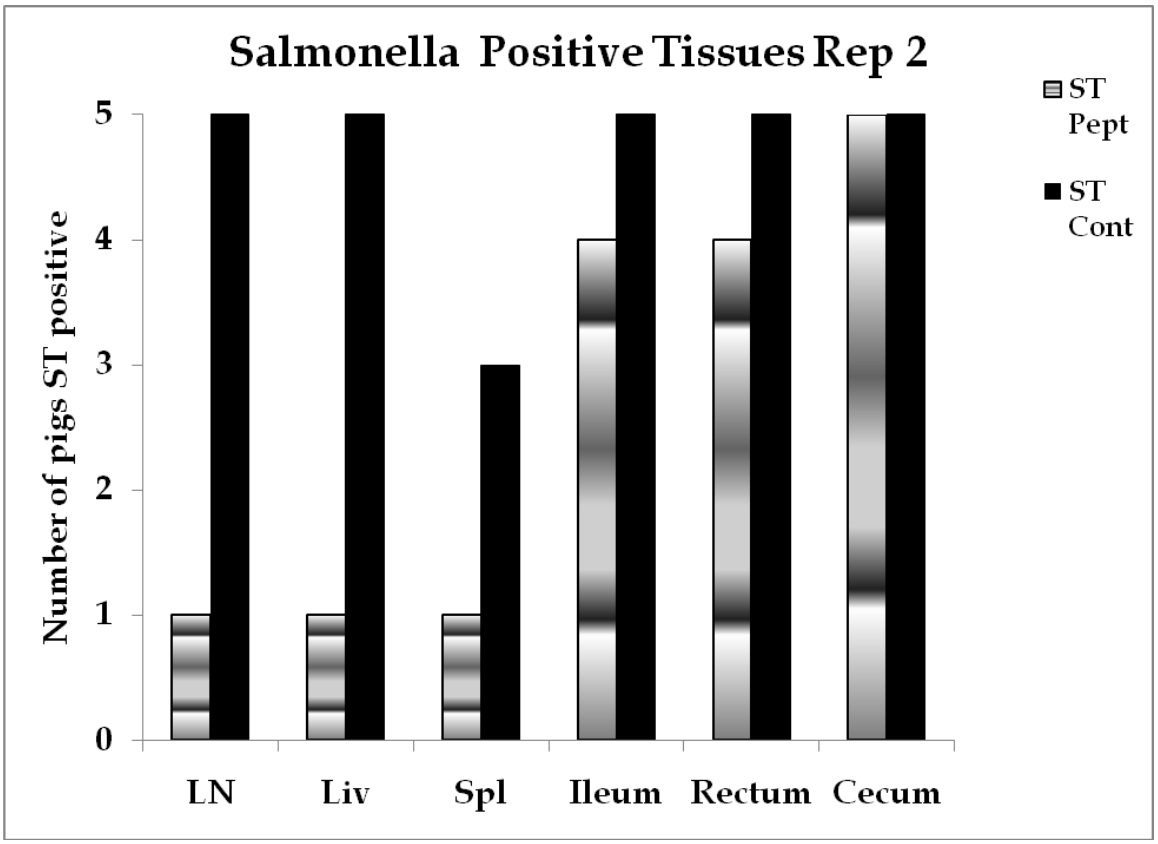




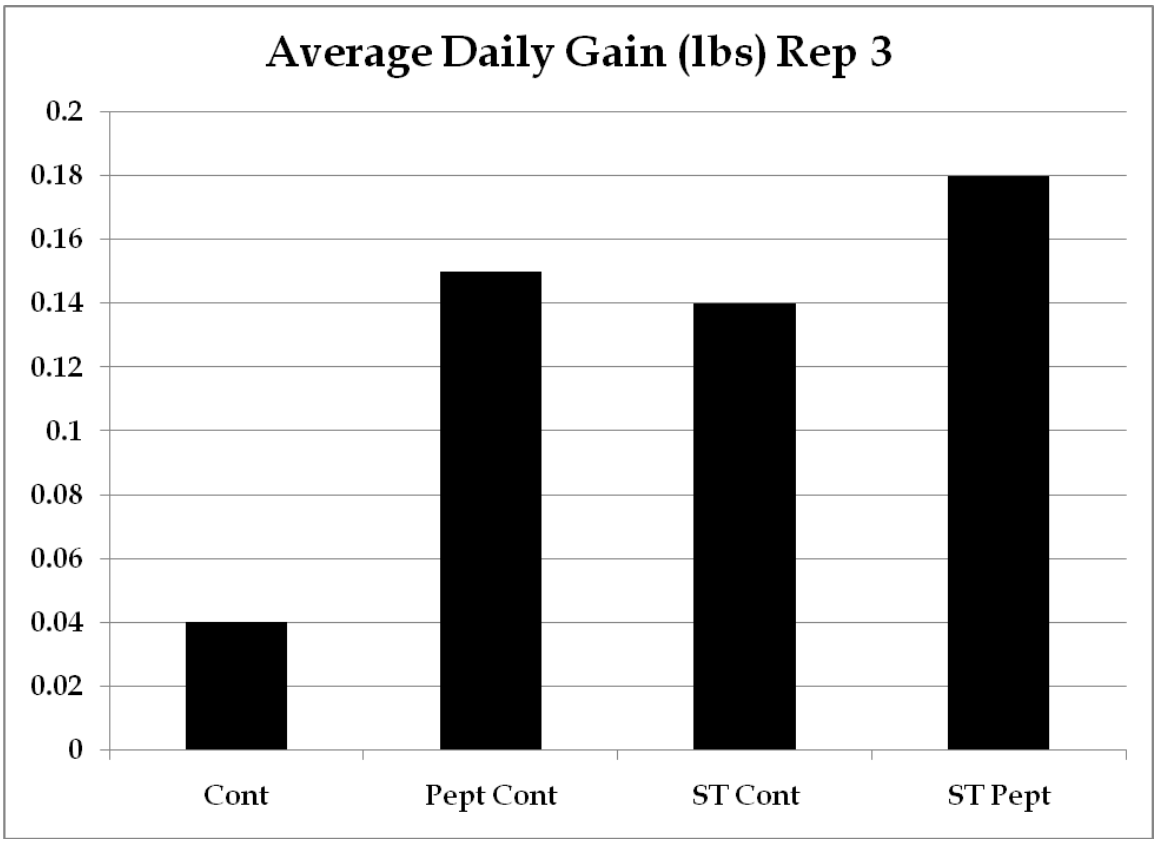
Rep 2 Data:

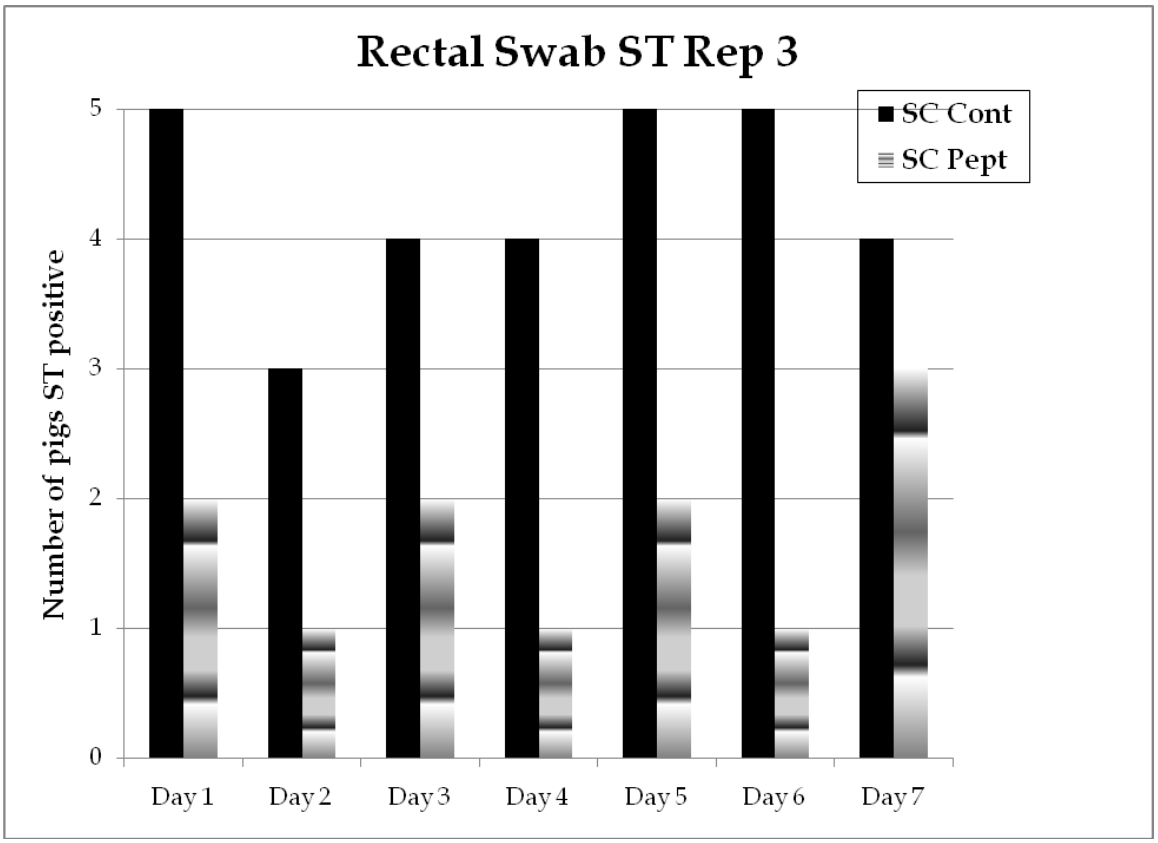

Average Daily Gain (lbs) Rep 2
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Cont
Cont Pept
ST Cont
ST Pept

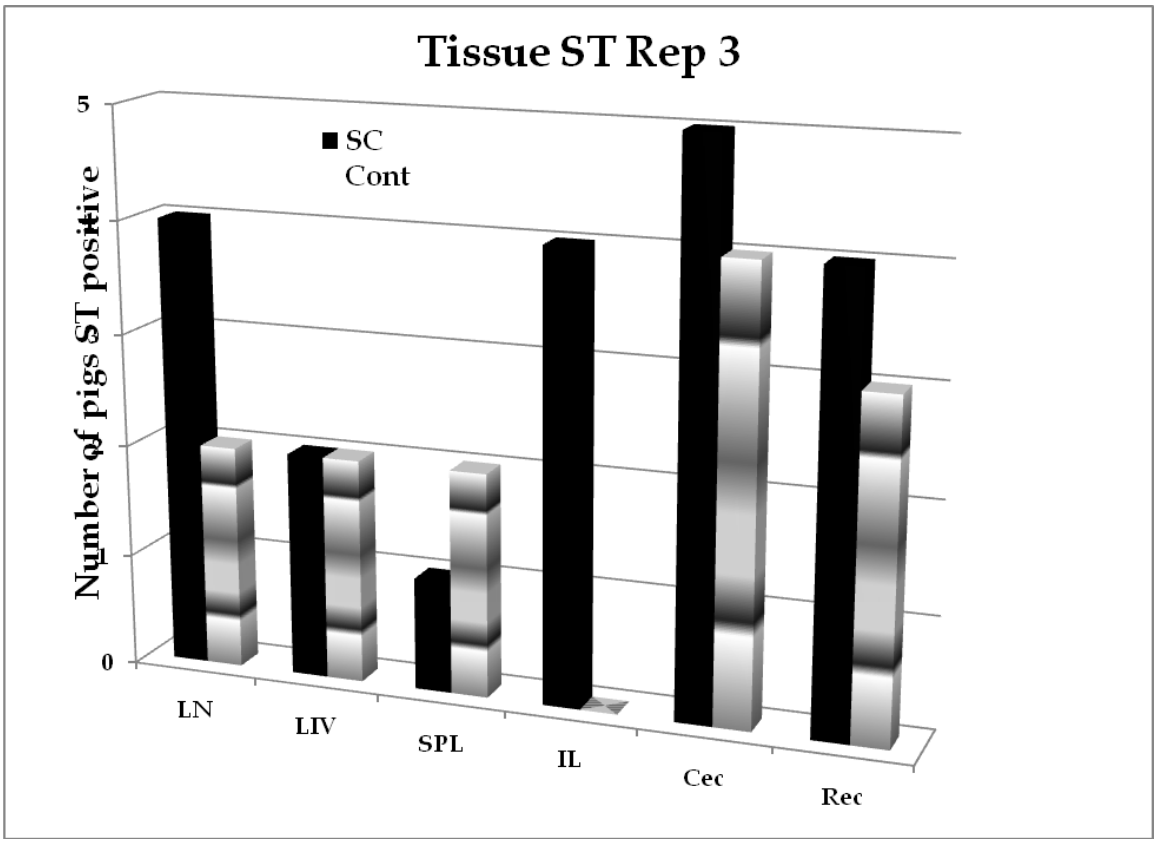

Daily Rectal Swab Data Rep 2
Number of pigs ST positive
0
1
2
3
4
5
Day 1
Day 2
Day 3
Day 4
Day 5
Cont ST


Salmonella Positive Tissues Rep 2
Number of pigs ST positive
0
1
2
3
4
5
LN
Liv
Spl
Ileum
Rectum
Cecum
ST Pept
ST Cont

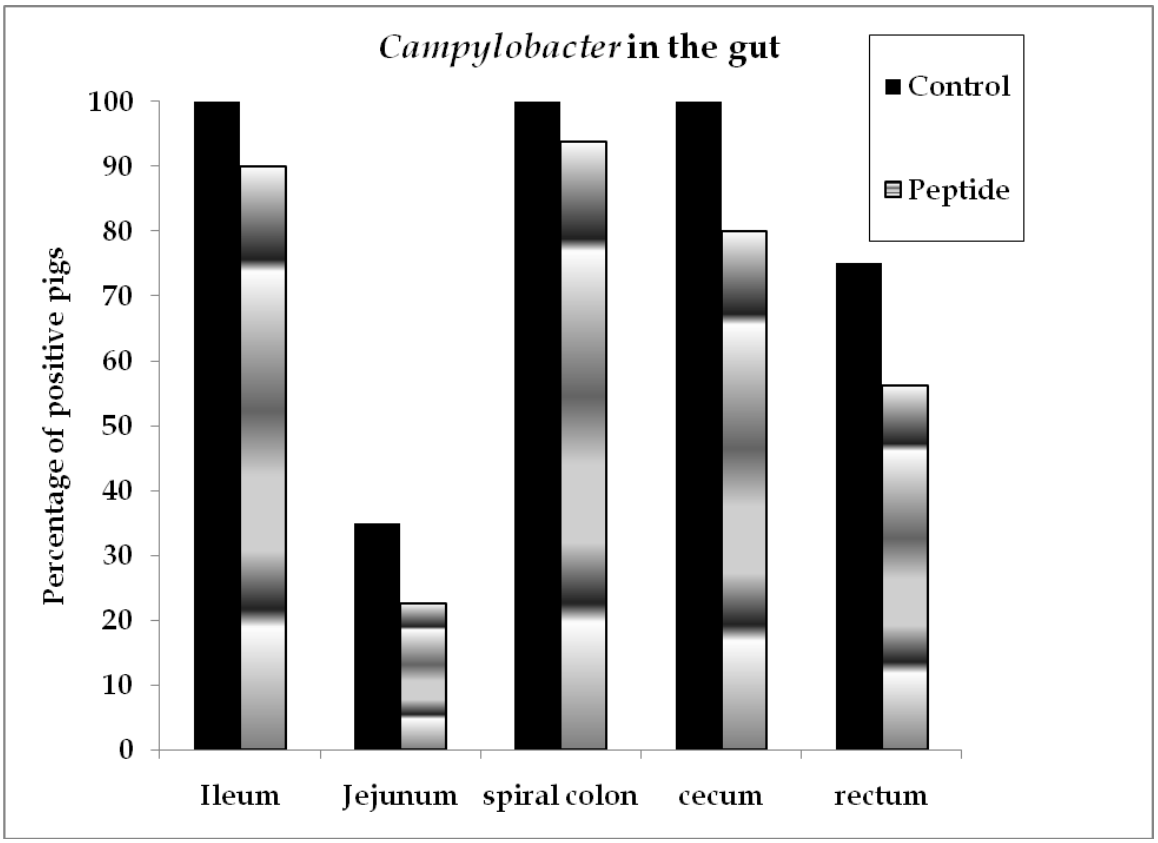
Rep 3 Data:


Average Daily Gain (lbs) Rep 3
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Cont
Pept Cont
ST Cont
ST Pept

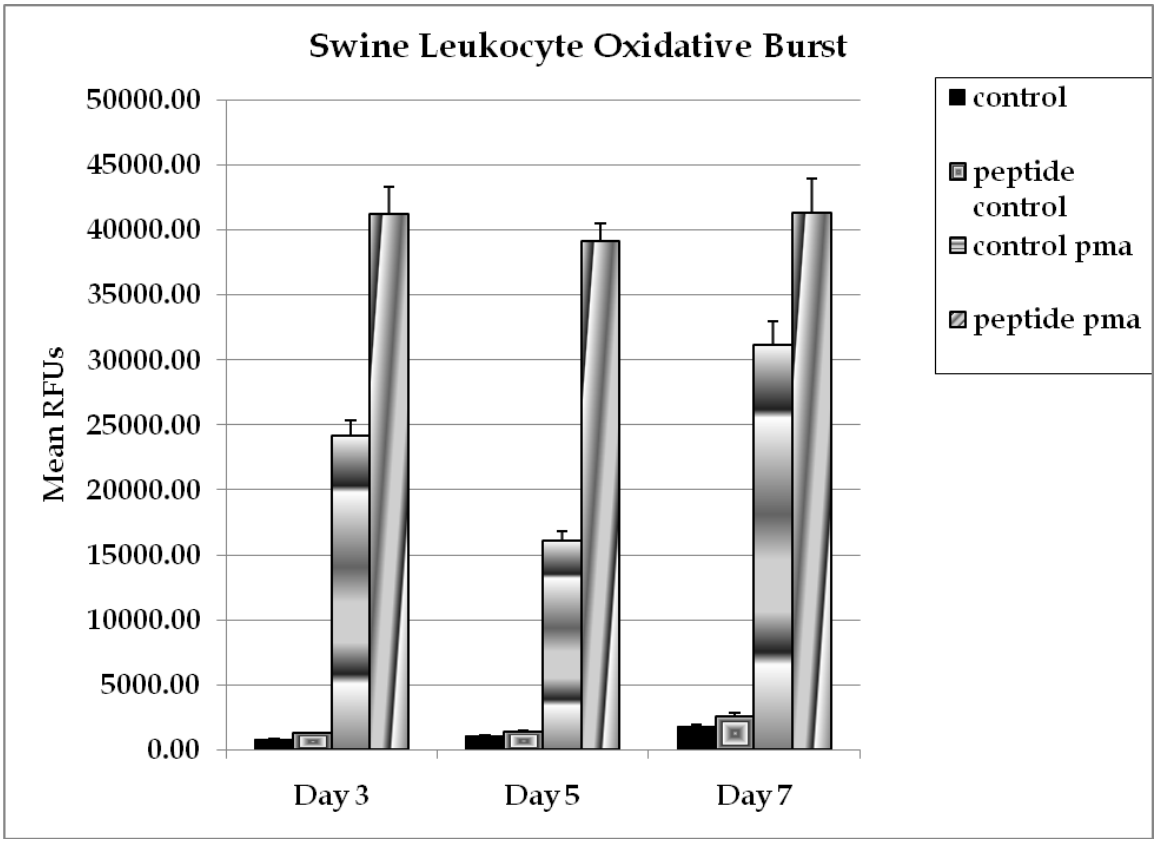

Rectal Swab ST Rep 3
SC Cont
SC Pept
Number of pigs ST positive
5
4
3
2
1
0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7



Campylobacter Results:

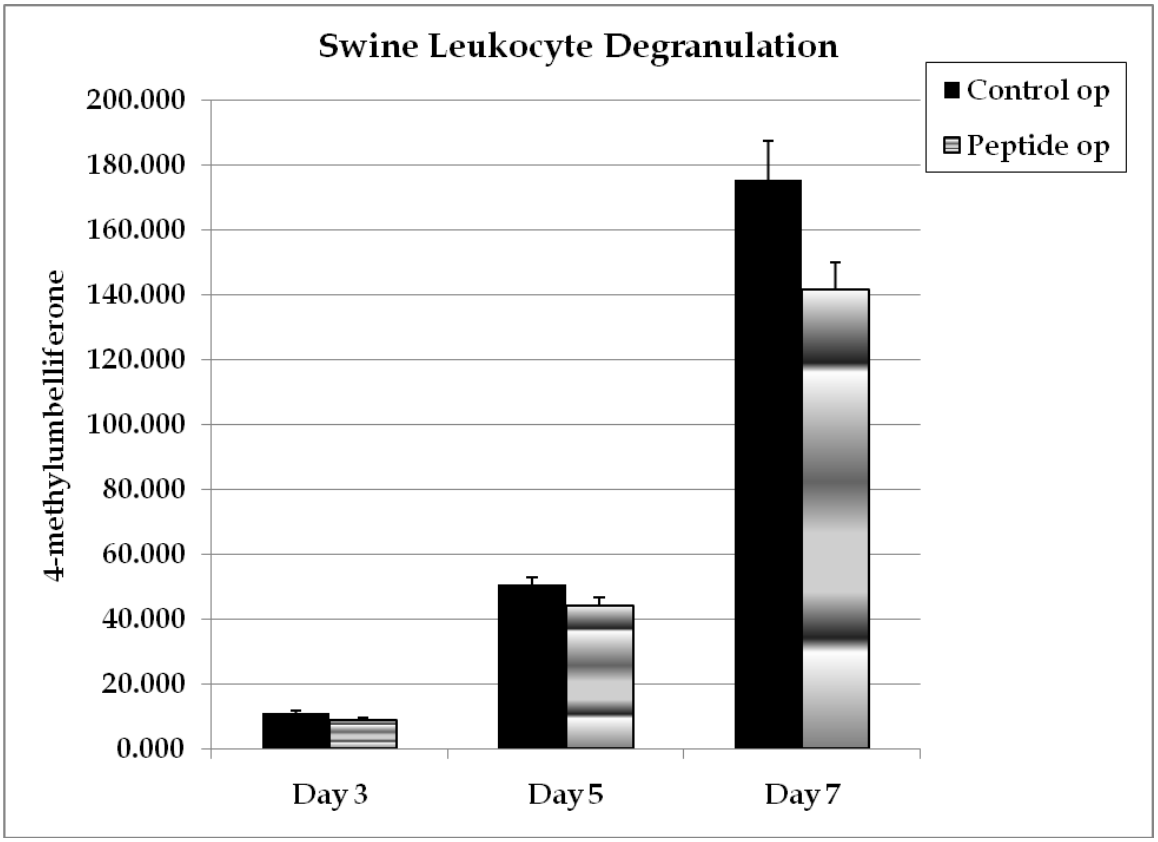


Objective 2, Immune response results



PMA = phorbol A-myristate 13 acetate
Op = opsonized *Salmonella typhimurium*

RFU = reflective fluorescent units



IX. Discussion:

The data presented here parallels work done in chickens with the BT peptide as a feed additive (Kogut et al., 2009). Pigs fed the peptide had increased immune functions, as shown by an increase in the oxidative burst response of leukocytes, and had decreased levels of *Salmonella* in tissues and the gut. No reductions in *Campylobacter* numbers in the gut of pigs fed the peptide during a preexisting infection were found.

The BT peptide represents an alternative intervention strategy to reduce the carriage and dissemination of *Salmonella* in swine to the use of antibiotics. The peptide is a "natural" product, derived from a bacteria, and its mode of action does not appear to be direct antimicrobial activity, but appears to involve immunomodulation of the innate immune system at the gut level. This may also explain why the peptide- fed pigs did not show reductions in *Campylobacter* colonization in preexisting infections in pigs due to the pig's tolerance of *Campylobacter* as a member of a normal flora. Although, if the pigs were fed the peptide for a longer period of time, perhaps reductions in *Campylobacter* would have been observed.

In conclusion, the BT peptide shows promise as a pre-harvest intervention strategy to prevent colonization of swine with food-borne pathogens and may have effects on other pathogens not studied here. The characteristic of being an immunomodulatory agent is significant because development of resistance to such an agent would not be expected. In addition, the BT peptide had a positive impact on weight gain, despite a *Salmonella* infection in weaned pigs. The production of the BT peptide could be readily scaled up for use in swine production making the peptide a contemporary product for the swine industry today.

REFERENCES

Bals, R. and Wilson, J.M. 2003. Cathelicidins - a family of multifunctional antimicrobial peptides. *Cell. Mol. Life Sci*. 60:711-720.

Bowdish, D.M.E, Davidson, D.J., Lau, Y.E., Lee, K., Scott, M.G., and Hancock, R.E.W. 2005. Impact of LL-37 on anti-infective immunity. *J. Leuk. Biol.*. 77:451-459.

Brogden, K.A., Ackermann, M., McCray, P.B., and Tack, B.F. 2003. Antimicrobial peptides in animals and their role in host defenses. *Int. J. Antimicrob. Agents* 22:465-478.

Finlay, B.B. and Hancock, R.E.W. 2004. Can innate immunity be enhanced to treat microbial infections? *Nature Rev. Microbiol*. 2:497-504.

Hancock, R.E.W. and Falla, T.J. 1996. Antimicrobial peptides: Broad-spectrum antibiotics from nature. *Clin. Microbiol. Infect*. 1:226-229.

Hoffman, J.A., Kafatos, F.C., and Janeway, C.A. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313-1318.

Jiang YW, Sims MD, Conway DP. 2005. The efficacy of TAMUS 2032 in preventing a natural outbreak of colibacillosis in broiler chickens in floor pens. *Poult Sci* 84:1857-1859.

Kogut MH, He H, Genovese KJ, Jiang YW. Feeding the BT cationic peptides to chickens at hatch reduces cecal colonization by Salmonella enterica serovar Enteritidis and primes innate immune cell functional activity. Foodborne Pathog Dis. 2010 Jan;7(1):23-30.
Wu X, Ballard J, Jiang YW. 2005. Structure and biosynthesis of the BT peptide antibiotic from *Brevibacillus texasporus*. *App. Environ. Microbiol*. 71:8519-8530
Yang, D., Biragyn, A., Hoover, D.M., Lubkowski, J., and Oppenheim, J.J. 2004. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Ann. Rev. Immunol*. 22:181-215.