RESEARCH REPORT
PORK SAFETY

Title: Toll-like receptor agonist administration for the prevention of *Salmonella* colonization of the swine gut - **NPB #06-003**

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II. Industry Summary:

The main objectives of this research project were to determine if the oral administration of certain novel innate ("natural") immune enhancers would protect piglets against *Salmonella* infections and if the oral administration of these immune enhancers had beneficial effects on some important immune functions in young piglets. One immune stimulant, CpG DNA (CpG), is a component of bacterial DNA that is recognized by the pig's immune system as a non-self threat and causes the pig to mount an immune response to the CpG DNA. By mounting a response to the CpG DNA, the pig's immune system is also then primed to take on other pathogenic microbes that it may encounter, such as *Salmonella*. The other immune stimulant, flagellin (Flag), is a protein produced by bacteria and is a component of the bacteria's flagella. Flagellin acts in much the same way as CpG on the pig's immune system. Each of these innate immune stimulants are recognized by the pig's immune system through members of a highly evolutionarily conserved family of receptors called the toll-like receptors (TLR). TLRs are now recognized as being highly important not only for initial encounters with most microbes, but are also important for the host to mount an acquired or "memory" response to pathogens during subsequent encounters.

For initial studies, pregnant sows were placed in farrowing crates at our facilities one to two weeks prior to farrowing. Upon farrowing, each litter from each sow was designated into one of six groups –

Group	Treatment
1	**control – no treatment, not infected**
2	**nonCpG controls, SC infected**
3	**FLAG, SC infected**
4	**CpG, SC infected**
5	**FLAG/CpG, SC infected**
6	**SC infected control**

Within 24 hours of birth, piglets were given their respective treatments via oral gavage. For one group of experiments, piglets were then challenged with *Salmonella choleraesuis* (SC) five days after birth. On day 10 after birth, piglets were euthanized and samples of gut and tissues were cultured using established techniques for the presence of SC. Reductions in fecal shedding, organ colonization, and gut colonization were observed in the CpG-, FLAG-, and CPG/FLAG combo-treated pigs as compared to the infected controls. In another set of experiments, piglets were treated as described above, but were kept on-sow until day 14 after birth. Piglets were weaned and then challenged with SC on Day 17. Piglets were then euthanized on Day 22 and cultured as described above. The results showed similar reductions in fecal shedding, organ colonization, and gut colonization observed in the CpG-, FLAG-, and CPG/FLAG combo-treated pigs as compared to the infected controls.

In a separate set of experiments, piglets were given their respective treatment at birth, but were not infected. Instead, piglets were bled at days 4, 7, 14, and 21 post-treatment and the biological functions of their blood leukocytes were assessed.

On Day 4, leukocytes from piglets in the flag, CpG, flag/CpG, and 2041 groups had higher levels of an oxidative burst, a measure of the leukocyte's ability to kill bacteria, than did piglets in the control group. On Day 7, only piglets in the flag/CpG group had higher levels of an oxidative burst than controls and other treatment groups. This trend continued on Days 14 and 21, with the flag/CpG leukocytes having higher oxidative burst levels than leukocytes from pigs in the other groups. Another form of microbial killing used by

leukocytes called degranulation was assessed on days 4, 7, 14, and 21 post-treatment. CpG, Flagellin, and the combination improved degranulation compared to control leukocyte degranulation on days 4, 7, 14, and 21. The combination group (CF) on day 21 had much higher cell degranulation than did all other groups.

The results of these experiments are encouraging and point to the possible use of CpG and flagellin either in combination or separately to combat pathogens in swine. Both CpG and flagellin had positive effects on the immune systems of pigs and on reducing Salmonella in pigs. In addition, studies in other species have pointed to the possible use of these agents as adjuvants for currently used and new vaccines to help boost the positive effects of these vaccines. Although currently these agents are expensive, it is believed that with mass-scale production, the price can be brought to a reasonable level. *Contact Information*: Kenneth J. Genovese; USDA-ARS, FFSRU, SPARC, College Station, TX 77845; genovese@ffsru.usda.gov; 979.260.3756.

III. Scientific Abstract:

Toll-like receptors (TLR) have been identified as important modulators of the innate immune response. Agonists for two of these receptors, TLRs 5 and 9, have been identified as being potent immunomodulators in avian and mammalian species. The purpose of the present studies was to evaluate the use of flagellin, a TLR 5 agonist, and CpG DNA, a TLR 9 agonist, and the combination of the two to reduce *Salmonella* colonization of the gut and organs of swine and determine if their administration to piglets had effects on innate immune functions of swine leukocytes. Piglets were treated with the agonists at birth by oral gavage, challenged with *Salmonella* 5 days later or after weaning, and then euthanized and cultured for the presence of *Salmonella*. Piglets treated with the agonists or the combination of the agonists had reductions in fecal shedding, organ invasion, and in gut contents of *Salmonella* as compared to control pigs both in pigs euthanized before and after weaning. In studies of the innate immune functions of swine leukocytes, piglets were bled and leukocytes isolated on days 4, 7, 14, and 21 post-treatment. Both flagellin and CpG and in combination were found to increase the oxidative burst and cellular degranulation of neutrophils isolated from treated pigs ($P < 0.001$).

These studies show that TLR agonists can be used on their own as innate immune stimulants and that these agonists had positive effects on *Salmonella* colonization in young pigs.

IV. Introduction:

Salmonella continues to be a food born pathogen of concern with over 40,000 cases reported each year by the CDC (2). Work by Hurd et al. has shown that pigs infected with *Salmonella* prior to entering the abattoir can pass the bacteria to other pigs during holding prior to slaughter and can become contaminated with serovars already present somewhere in the holding facilities (11,12). The carriage of *Salmonella* then into the slaughter facilities presents an opportunity for the contamination of pork products. The prevention of colonization and carriage of *Salmonella* by pigs prior to slaughter is an important point of control for this food born pathogen. However, preharvest control of a pathogen such as *Salmonella* is difficult due to the many possible points of contamination during the production life of a pig and the multiple serovars involved. Controlling *Salmonella* early in the pig's life may be one way to decrease the exposure of pigs to *Salmonella* and subsequently the number of pigs entering the abattoir carrying *Salmonella*.

Research has shown that the immune systems of young pigs as well as other animals do not fully mature until after weaning. Although colostrum represents an avenue for the protection of suckling pigs against colonization and infection with pathogens, this defense can and does fail at times to provide protection. One reason for the failure of colostrum to prevent the colonization of suckling pigs with pathogens may be that the sow lacks previous exposure to a particular pathogen and subsequently cannot pass along antibodies to protect the piglet from the pathogen. This need for specificity in the antibody-mediated, acquired immune response places the piglets in a susceptible situation where an encounter with a pathogen that the sow has not previously been exposed to allows the piglet to become colonized and infected. The possibility of boosting the non-specific innate immune response provides a strategy for enabling the young piglet to defend itself against all bacterial pathogens, including those the sow has not been exposed to. In addition, the innate immune response

aids in the direction and the engagement of the immune components necessary for the inception of an acquired immune response (16-18).

The innate immune arm of the immune response is the first line of defense the host uses to defend against invading microbes. The innate system is the primary responder to initial contact with microbes, responsible for the initial control and elimination of microbial invaders that have breached the host's physical defenses. It is through this initial interaction that the host engages the immune machinery necessary for immediate host defense and the development of an acquired response for long-term protection against re-infection with microorganisms. An important part of the recognition of pathogens by the innate immune system is mediated by receptors on cells of the immune system called pattern recognition receptors (PRR) (13-15). These PRRs recognize unique molecular structures of pathogens. Members of these receptors include the Toll-like receptors (13). Toll-like receptors (TLR) are important mediators of the host innate immune response to bacterial and viral infections and a total of 11 TLR have been identified to date (13). TLR recognize highly conserved antigens of bacterial and viral invaders called pathogen-associated molecular patterns (PAMPS) (1,3,14). These PAMPS are categorized according to the pathogen and can be sequences of bacterial DNA, viral DNA, or proteins or other components associated with the pathogen. Binding of the receptor with a PAMP initiates a cascade of intracellular signaling events that leads to the host's response to the invading microbe including cell activation, gene transcription, and cytokine release (13-15). Bacterial flagellin (FLAG), a protein that is part of the flagellar apparatus, has been found to stimulate an innate immune response through its recognition by TLR 5 (15). FLAG has been shown to be an effective stimulant of the innate immune response in mice when administered intra-bronchially (10). However, much of the research on FLAG as an immunostimulatory agent has been done *in vitro*. Bacterial DNA can also stimulate the innate immune system through a TLR. The bacterial genome contains pieces of DNA that include specific sequences of unmethylated bases termed CpG DNA (15). CpG DNAs have been identified as ligands for TLR 9 (15).

V. Objectives:

1. To ascertain the effectiveness of the oral administration of TLR agonists to neonatal swine in the prevention of gut and tissue colonization by *Salmonella* during the suckling period and post-weaning.

2. Determine the effects of TLR agonists on the innate immune functions in neonatal swine over time when administered at birth. Specifically, identify the effects of TLR agonist administration on neutrophil and monocyte functions from birth until the post-weaning period.

VI. Material and Methods:

Infection studies: Pregnant sows were obtained from a commercial source one to two weeks prior to farrowing. Sows were housed in farrowing crates and have *ad libitum* access to feed and water. The initial experiments involved two sets of sows (set A and set B), with 7 sows/set**.** One litter per set was used to provide piglets for those sows that did not have 10 healthy, viable piglets in a litter.

- *Experimental design for set A*: Sows were selected from the commercial source to have farrowing dates as close to the same day as possible. Upon farrowing, the litter the was placed into one of six groups. Piglets remain on-sow for the duration of the experimental period.

Group	Treatment
1	**control – no treatment, no infected**
2	**nonCpG controls, SC infected**
3	**FLAG, SC infected**
4	**CpG, SC infected**
5	**FLAG/CpG, SC infected**
6	**SC infected control**

Piglets in the CpG (**4, 5**) and nonCpG control (**2**) groups received 1.0 mg of their respective treatment in 2ml of phosphate-buffered saline (PBS) by oral gavage. Piglets in the FLAG groups (**3, 5**) received 1.0 mg of FLAG in 2 ml PBS by oral gavage. Pigs in the nontreated, noninfected (**1**) and SC infected control (**6**) groups received an oral dose of 2ml PBS. Piglets were weighed, ear-tagged, and given their respective treatment within 24 hours after birth. The day the treatment is administered was designated day 0. On day 5 post-treatment, all piglets were weighed; piglets in all infection groups were orally administered 2 ml of phosphate-buffered saline solution (PBS) containing 10^9 colony-forming units (cfu) of *Salmonella choleraesuis* (SC). Daily rectal swabs were taken from all groups and cultured for the presence of SC following established procedures (6). On day 10

post-treatment, pigs were weighed, then euthanized and sections of the gut and lymph nodes were cultured for the presence of SC (6). The cecum, colon, and rectum tissues and the luminal contents were cultured for the presence and enumeration of SC, respectively. In addition, the liver, spleen, and ileocecal lymph nodes were cultured for the presence of SC. Methods in (6).

- *Experimental design for set B:* Litters from sows in set B were treated as in set A, with the following changes. Piglets received their respective treatments on day 0 (within 24 hours of birth) as in set A. In set B, however, piglets remained on the sow without being infected until day 14. Piglets were weighed on day 7. On day 14 piglets were weighed and placed into floor pens (1 litter/treatment group/pen) with *ad libitum* access to a phase I diet and water. On day 17, piglets were orally challenged with 4 ml of 10^9 cfu SC. Rectal swabs were taken daily. On day 22, piglets were weighed and then euthanized. Gut contents and tissues were cultured as stated previously.

In vitro assessment of TLR agonist administration: In these experiments, piglets in litters from sows were treated as in the infection studies described above. However, no infections were introduced.

- *Experimental design*: Piglets born to each sow were placed into one of six groups. Piglets received their respective treatments within 24 hours of birth (day 0). On days 4, 7, 14, and 21 peripheral blood samples were obtained from 5 piglets in each group and pooled. Neutrophils and monocytes 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. Macrophage production of bactericidal nitric oxide will also be assessed. Briefly, the assays are described below.

Degranulation will be detected by quantifying the amount of β-glucuronidase activity in the culture medium following stimulation of neutrophils (8×10^6 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].

Neutrophil oxidative burst will be measured by oxidation of DCFH-DA to fluorescent DCF as described by He et al., (9). Neutrophils 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].

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 SE: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 SE and the average number of SE/neutrophil.

Nitric oxide production: Cells are placed (1 x10^7 cells/ml) into a 96-well plate and incubated at room temperature for 2 hr. After incubation, non-adherent cells are removed by washing twice with culture medium. Adherent monocytes in 200µl of media will then be stimulated with an agonist for 48 hr at 41ºC in 5% CO_2, 95% humidity in an incubator. Nitrite by activate monocytes will then be measured by the Greiss assay.

Briefly, 100 µl aliquot of culture supernatant from each well is transferred to a new 96-well plate and combined with 1% sulfanilamide and 50 µl of 0.1% napthylenediamine. After 10 min incubation at room temperature, the nitric concentration will be determined by measuring optical density (OD_{595}) of each well using a SPECTRA MAX microplate reader (Molecular Devices). Sodium nitrite is used as a standard to determine nitrite concentrations in cell-free medium.

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.

VII. Results:

Experiments from Sow set A and Sow set B are presented below. Under conditions for both sets, reductions in fecal shedding, organ colonization, and gut colonization were observed in the CpG-, FLAG-, and CPG/FLAG combo-treated pigs as compared to the infected controls. This data is encouraging and the immunological experiments planned for Objective 2 will aid in the understanding of the protection against *Salmonella* colonization observed in experiments for Objective 1.

Table 1: Set A: Enumeration of *Salmonella*/gram of gut contents

Group	Colon	Cecum	Rectum
CpG	1.50×10^4 cfu/g†	1.60×10^4 cfu/g	0.00
SC control	3.77×10^5cfu/g	1.95×10^5cfu/g	3.47×10^3cfu/g
Flag	5.10×10^3 cfu/g	1.12×10^3 cfu/g	1.20×10^2 cfu/g
Flag/CpG	1.85×10^4 cfu/g†	1.40×10^3 cfu/g	1.14×10^2 cfu/g
NonCpG	2.25×10^5cfu/g	1.90×10^3 cfu/g	2.80×1010^3cfu/g

† cfu = colony forming units

Figure 1: *Salmonella* isolated from tissues

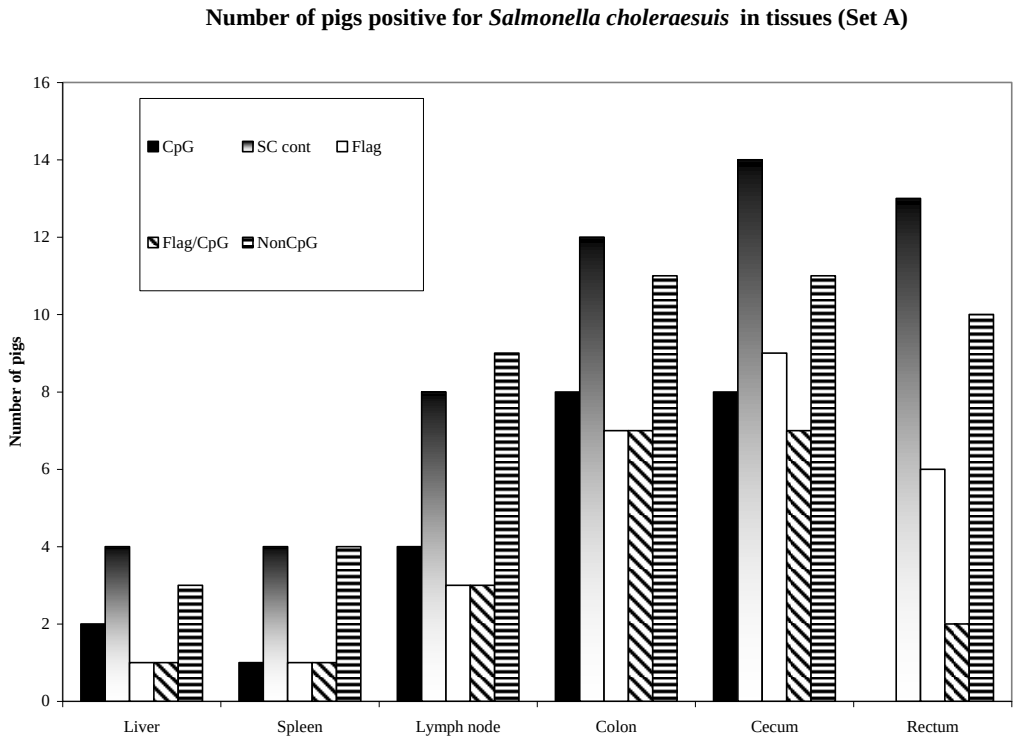


Figure 2: Rectal swab data – Set A

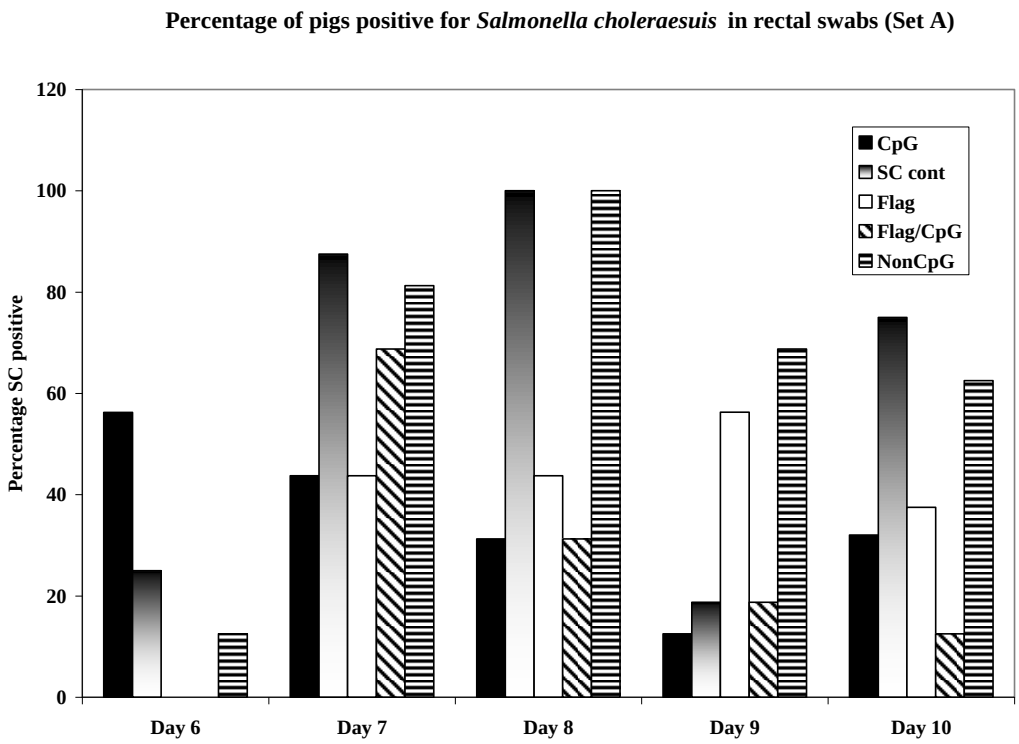


Table 2: Set B: Enumeration of *Salmonella*/gram of gut contents (cfu/g)

Group	Colon	Cecum	Rectum
CpG	0.00	3.0×10^2	0.00
SC cont	1.80×10^3	5.0×10^3	4.80×10^2
Flag	2.60×10^3	1.32×10^2	3.70×10^2
CpG/Flag	2.85×10^3	2.00×10^2	1.60×10^2
NonCpG	4.10×10^3	6.32×10^3	2.30×10^2

Figure 3: Weights- Set B

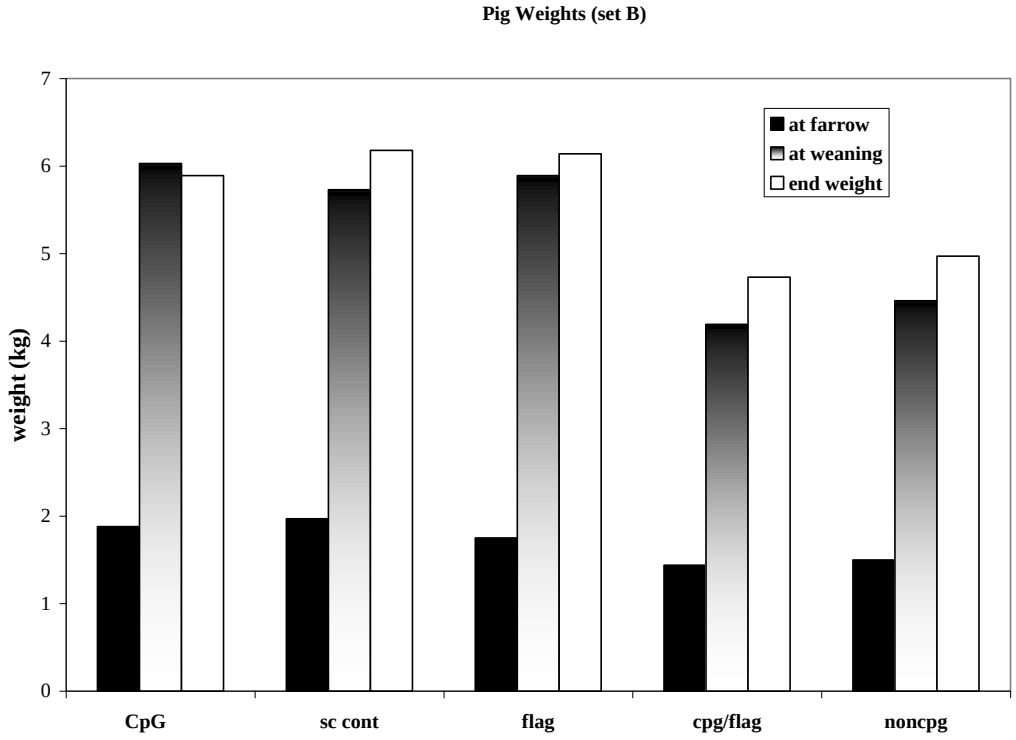


Figure 4: *Salmonella* isolated from tissues – Set B

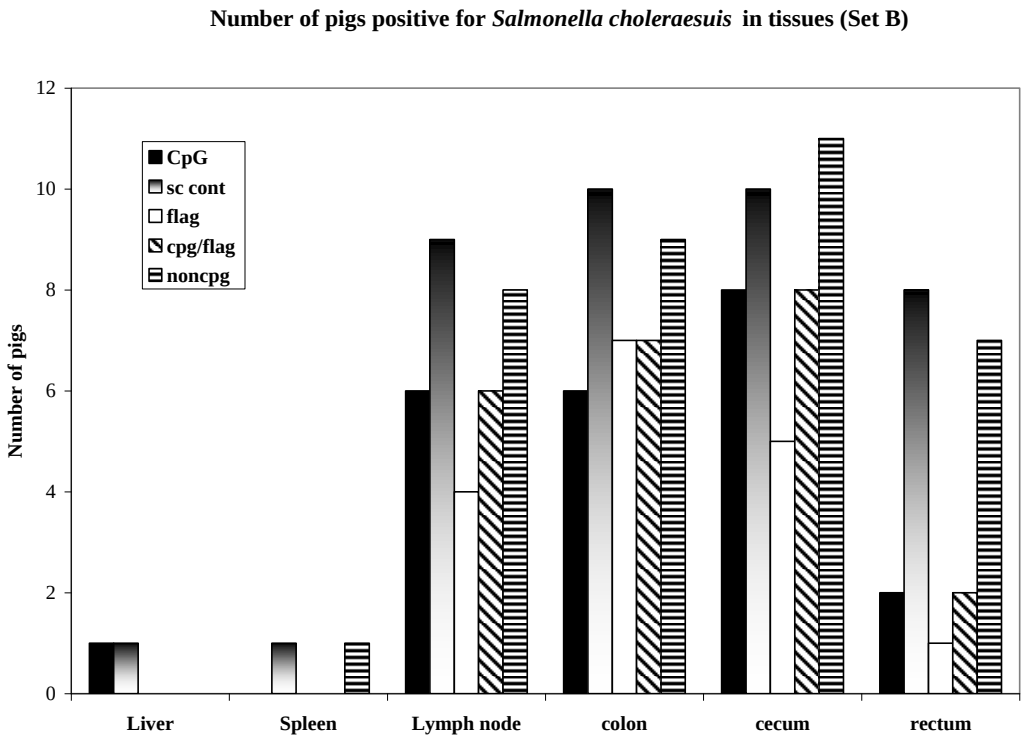
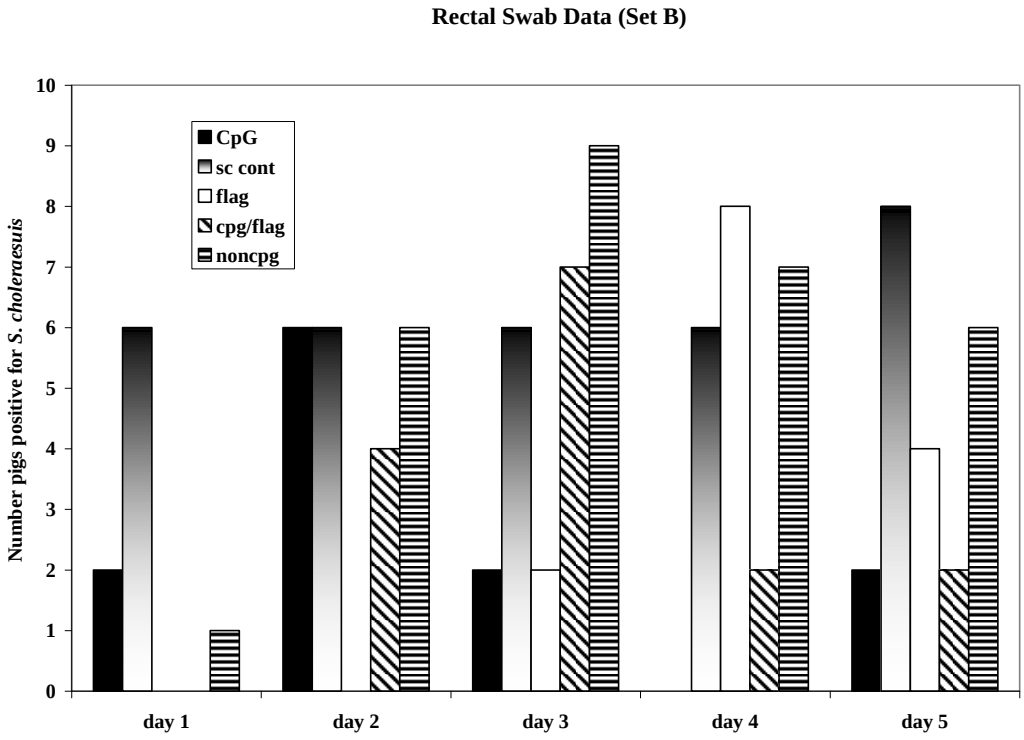


Figure 5: Rectal swab data – Set B



Results for Objective 2.

Below the data for Objective 2 is presented. In figures 1-4, the data for neutrophil oxidative burst is presented. On Day 4, neutrophils from piglets in the flag, CpG, flag/CpG, and 2041 groups had higher levels of an oxidative burst than did piglets in the control group. On Day 7, only piglets in the flag/CpG group had higher levels of an oxidative burst than controls and other treatment groups. This trend continued on Days 14 and 21, with the flag/CpG neutrophils having higher oxidative burst levels than leukocytes from pigs in the other groups. In Figure 5, neutrophil degranulation data is presented. Degranulation was assessed on days 4, 7, 14, and 21 post-treatment. CpG, Flagellin, and the combination improved degranulation compared to control neutrophil degranulation on days 4, 7, 14, and 21. The combination group (CF) on day 21 had much higher cell degranulation than did all other groups. Due to limited cell numbers isolated from the piglets, phagocytosis was not performed. Swine monocyte production of nitric oxide was found to be lacking. No nitric oxide was found to be produced when cells were incubated with stimulants as described in the materials and methods section. This is, however, in concurrence with reports by other researchers which suggests that swine monocytes do not produce significant levels of NO in their innate immune response (19).

Figure 1:

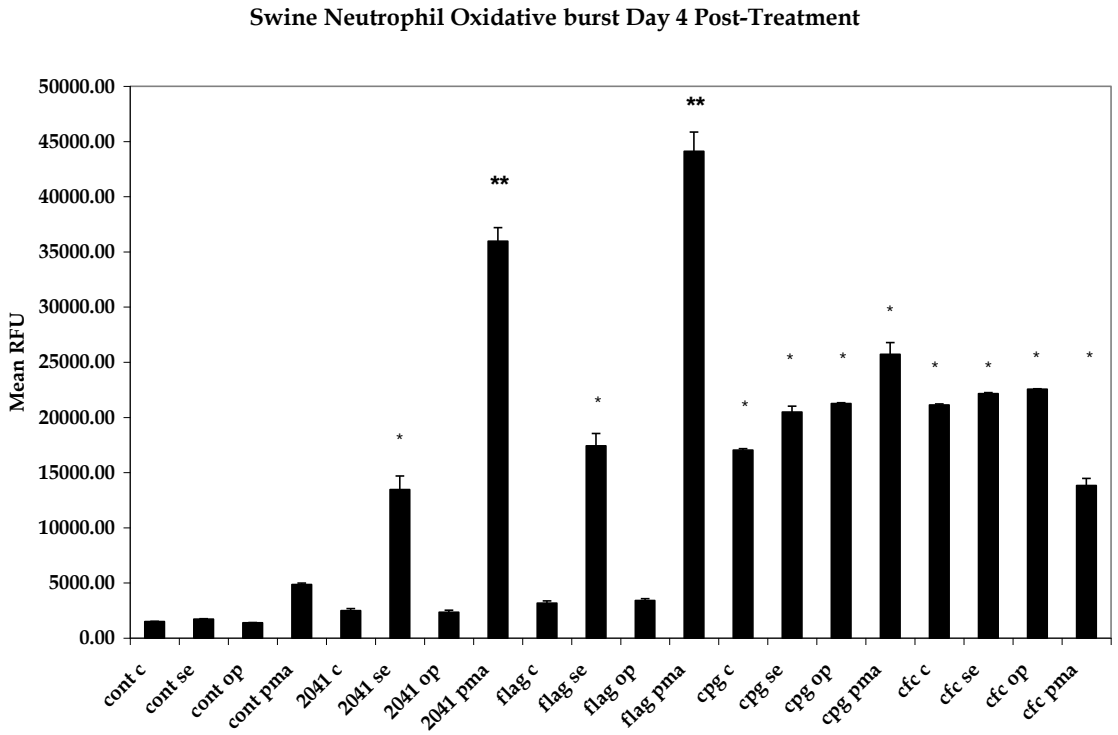

Swine Neutrophil Oxidative burst Day 4 Post-Treatment
Mean RFU
50000.00
45000.00
40000.00
35000.00
30000.00
25000.00
20000.00
15000.00
10000.00
5000.00
0.00
cont c
cont se
cont op
cont pma
2041 c
2041 se
2041 op
2041 pma
flag c
flag se
flag op
flag pma
cpg c
cpg se
cpg op
cpg pma
cfc c
cfc se
cfc op
cfc pma

Figure 2:

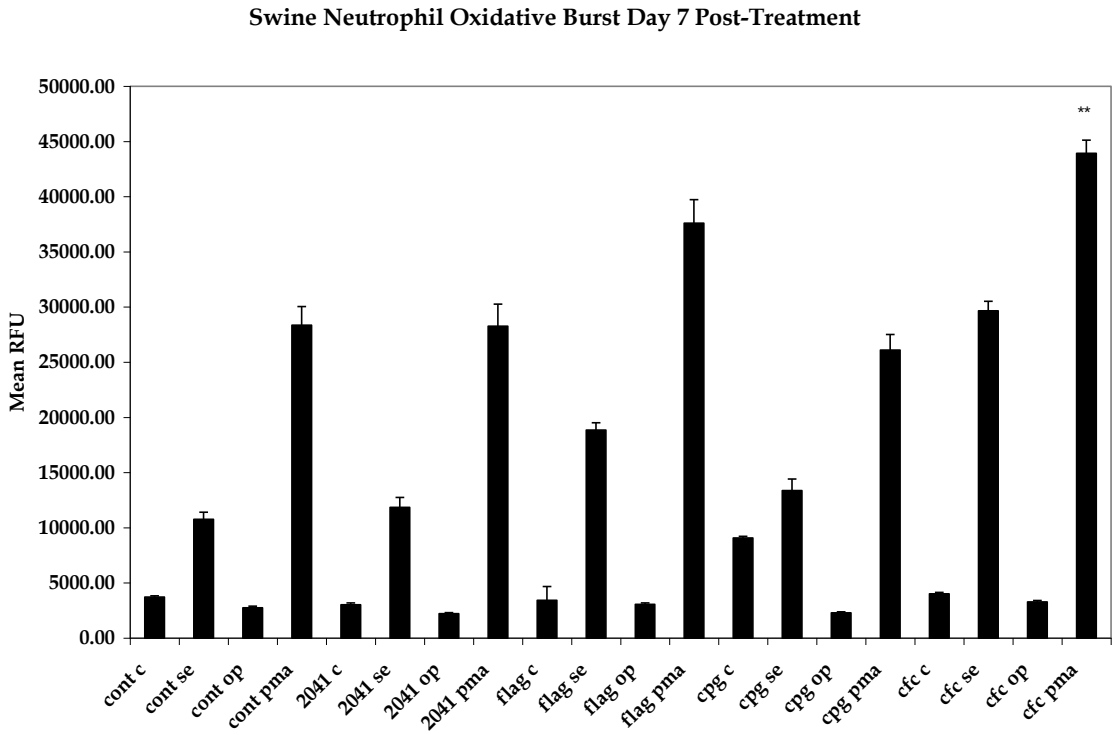

Swine Neutrophil Oxidative Burst Day 7 Post-Treatment
Mean RFU
50000.00
45000.00
40000.00
35000.00
30000.00
25000.00
20000.00
15000.00
10000.00
5000.00
0.00
**
cont c
cont se
cont op
cont pma
2041 c
2041 se
2041 op
2041 pma
flag c
flag se
flag op
flag pma
cpg c
cpg se
cpg op
cpg pma
cfc c
cfc se
cfc op
cfc pma

Figure 3:

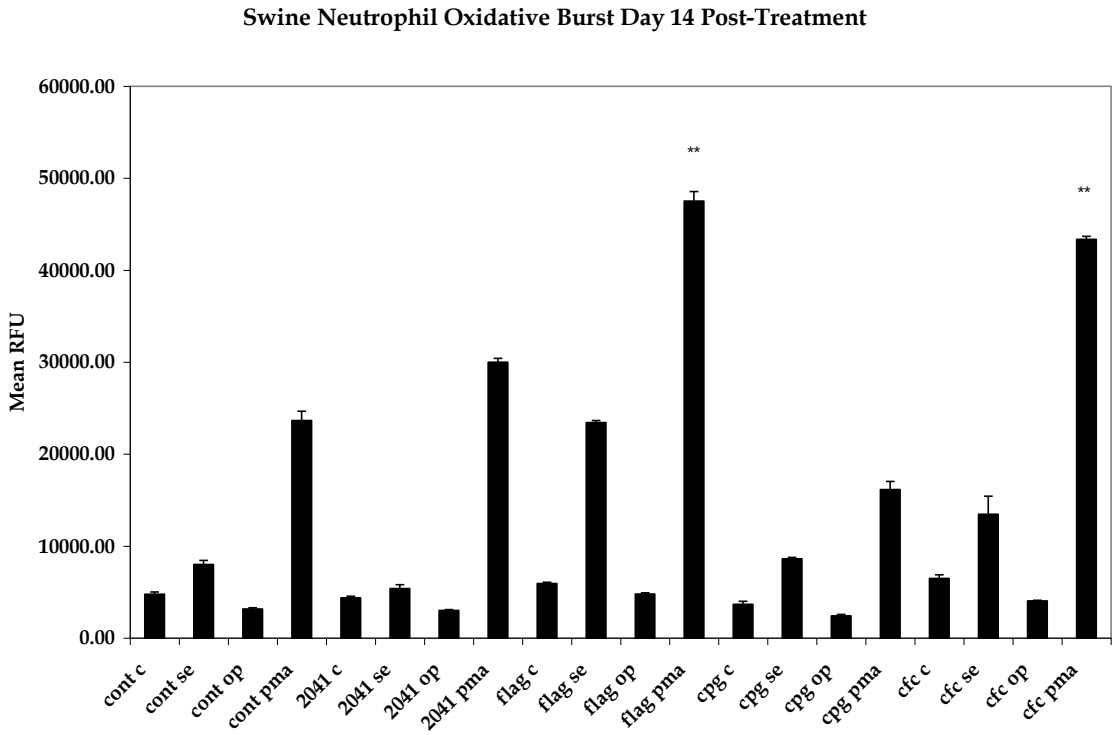

Swine Neutrophil Oxidative Burst Day 14 Post-Treatment
Mean RFU
60000.00
50000.00
40000.00
30000.00
20000.00
10000.00
0.00
**
**
cont c
cont se
cont op
cont pma
2041 c
2041 se
2041 op
2041 pma
flag c
flag se
flag op
flag pma
cpg c
cpg se
cpg op
cpg pma
cfc c
cfc se
cfc op
cfc pma

Figure 4:

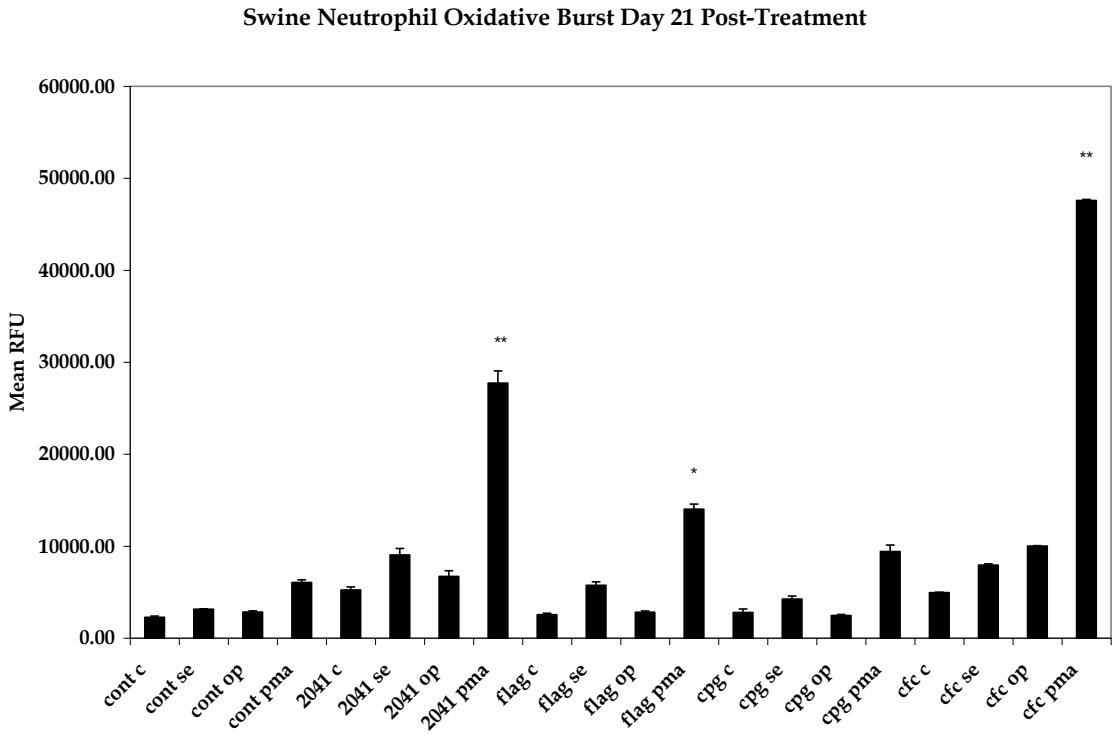
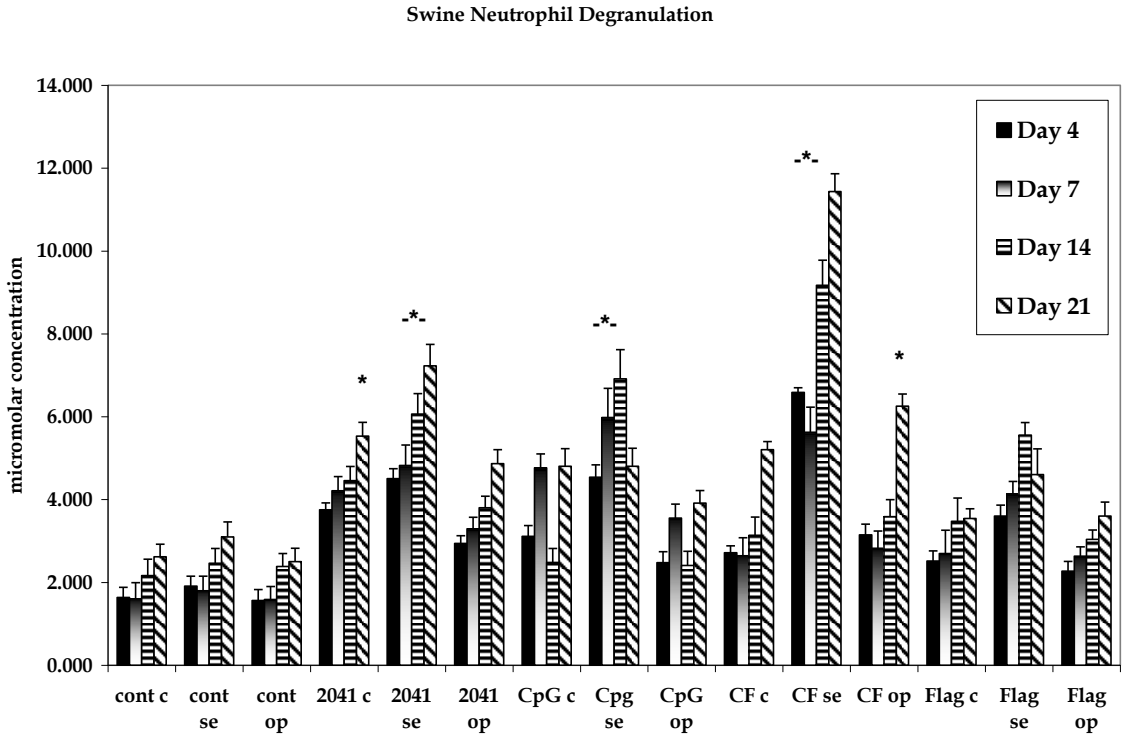

Swine Neutrophil Oxidative Burst Day 21 Post-Treatment
Mean RFU
60000.00
50000.00
40000.00
30000.00
20000.00
10000.00
0.00
**
*
**
cont c
cont se
cont op
cont pma
2041 c
2041 se
2041 op
2041 pma
flag c
flag se
flag op
flag pma
cpg c
cpg se
cpg op
cpg pma
cfc c
cfc se
cfc op
cfc pma

Figure 5:


Swine Neutrophil Degranulation
micromolar concentration
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
Day 4
Day 7
Day 14
Day 21
*
-*-
-*-
-*-
*
cont c
cont se
cont op
2041 c
2041 se
2041 op
CpG c
Cpg se
CpG op
CF c
CF se
CF op
Flag c
Flag se
Flag op

VIII. Discussion:

The results presented in this report point to the use of Toll-like receptor agonists as possible immunopotentiators in swine. Reductions were noted in the number of *Salmonella* found in the gut as well as reductions in fecal shedding and organ invasion. In addition, during the work for Objective 2 , it was found that the oral administration of the TLR agonists stimulated the immune system of piglets, in some cases for up to 21 days post-treatment. The results are highly encouraging and the use of these immunopotentiators in swine should be further investigated. The Investigator believes that the most probable use of these agents will be as vaccine adjuvants, boosting the pig's response to vaccines currently used in production as well as new vaccines that come on the market. Recent evidence suggests that the innate immune system plays a direct role in the development of an effective and appropriate acquired immune response and it is believed the role in vaccination responses is extremely important.

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.

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