RESEARCH REPORT
SWINE HEALTH

Title: The Use of Probiotics as an Aid in the Control of Clostridium difficile Associated Disease in Neonatal Pigs - **NPB #12-188**

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Date Submitted: February 28, 2014

Industry Summary:

In the last 10 years, *Clostridium difficile* has been implicated as a major cause of neonatal diarrhea in pigs. *C. difficile* infection (CDI) affects piglets ranging in age from 1-7 days. Clinical signs of CDI include diarrhea, abdominal distention and scrotal edema with most of the pathology being attributed to toxin production by this bacteria (Toxin A and B). Currently there are no commercial vaccines against *C. difficile* and the use of prophylactic antibiotics has been unsatisfactory and unrewarding for swine producers. *Clostridium difficile* infection is currently considered one of the most common causes of nosocomial diarrhea in humans. Therefore, we hypothesize that early oral administration of probiotics to pigs will control disease after experimental infection with *C. difficile*. An economical, safe, and simplistic therapy to control CDI and its subclinical incidence could be very beneficial to the swine industry.

Four replicates of the experimental design were conducted involving a total of 150 new born, caesarian derived piglets. Each experiment (replicate) divided piglets into 6 different study groups. GROUP 1 negative control, GROUP 2 piglets only received non-toxigenic *C. difficile* strain, GROPU 3 piglets received only probiotic yogurt, GROUP 4 positive control (challenged with toxigenic *C. difficile* strain), GROUP 5 animals received the probiotic non-toxigenic *C. difficile* strain and then challenged with the toxigenic *C. difficile* strain, GROUP 6 received probiotic yogurt and then challenge with the toxigenic *C. difficile* strain. Piglets were individually housed with non-challenged piglets (Groups 1 -3) being house in separate airspace than challenged piglets (Groups 4-6). The challenge isolate originated from a field case of neonatal diarrhea in 3-6 day-old piglets with high levels of toxin detected. Piglets were fed a set amount of milk replacer via gastric intubation three times daily. All pigs were humanely euthanized 72 hours post-challenge. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee.

At necropsy, gross observations at necropsy included 1) body condition, 2) dehydration status, 3) perineal fecal staining, 5) consistency of colonic contents, 6) mesocolonic edema, and the presence of 7) visible colonic luminal necrosis and were scored independently in a blinded fashion as previously described. Necropsies, clinical sign scores and gross lesion scores were completed by the same two individuals for all experiments. Fresh and formalin fixed tissues including ileum, jejunum, descending colon, cecum, and a cross section of spiral colon were collected. All tissues were submitted for histopathologic examination by a veterinary pathologist that was blinded to animal group designation. Rectal swabs collected prior to inoculation and pooled colon and cecum contents collected at necropsy were assayed for *C. difficile* toxins using a commercially available toxin ELISA kit providing a semi quantitatively measure of the amounts of toxin from 0 (no toxin detection) to 4+ (marked toxin detection) as indicated by the manufacturer.

For statistical analysis, three categories of scores were compared: 1) clinical signs, 2) ELISA results and mesocolonic edema, and 3) microscopic lesions. Clinical signs scores were created by summing scores for body condition, hydration status, and perineum staining. Microscopic lesion score was the sum of scores for all histopathology categories as previously described.

Overall statistical evaluation of clinical signs scores from study groups revealed no statistical difference ($P>0.05$). Statistical analysis revealed an overall significant difference in mesocolonic edema scores ($P=0.01$). Group 4 presented significant higher scores when compared to Groups 1, 2 and 5 ($P=$ 0.04, 0.018 and 0.002 respectively). All animals were ELISA negative at the beginning of the experiment. At necropsy (72 hours post inoculation), Group 5 had significant lower levels of toxin A and B detected via ELISA in colonic content when compared to Groups 3, 4 and 6. Histopathologic examination revealed classic CDI microscopic lesions characterized by variable numbers of intestinal only observed within colon and cecum. Microscopic scores were statistically different between groups (P=0.03). Group 4 had significant higher scores when compared to Groups 2 (P=0.005) and 5 (P=0.02). No other pairwise comparisons were significantly different. Analysis of data indicated that mesocolonic edema, ELISA toxin levels and histologic lesions are highly and significant correlated (P=0.001).

This project demonstrated that the use of non-toxigenic *C. difficile* probiotic was effective in minimizing the histologic lesions associated with *C. difficile* infection. Interestingly this decrease was also seen in the non-challenged piglets that received this non-toxigenic probiotic. The *Lactobacillus spp* probiotic also trended to have slightly lower histologic lesions than Groups 5 and 6 although statistical significance was not reached. It could be that for the *Lactobacillus spp* probiotic a continuous daily dose may be necessary to provide effective protection against CDI. It was also very interesting that for the first time, to our knowledge, we were able to see a direct association between mesocolonic edema, toxin levels and histologic lesions. This supports our current understanding of CDI as a toxin mediated disease. It is very feasible that non-toxigenic C. difficile probiotic can be easily used in the field to mitigate CDI in newborn piglets.

Keywords: probiotics, *Clostridium difficile*, diarrhea, neonatal, enteric, non-toxigenic, *Lactobacillus spp*

Scientific Abstract:

The effect of the use of early oral administration of probiotics, either a non-toxigenic *Clostridium difficile* or *Lactobacillus spp*, to newborn piglets would control disease after experimental infection with *C. difficile*. Four replicates of the experimental design were conducted involving a total of 150 new born, caesarian derived piglets. Each replicate divided piglets into 6 different study groups. GROUP 1 negative control, GROUP 2 piglets only received non-toxigenic *C. difficile* strain, GROPU 3 piglets received only probiotic yogurt, GROUP 4 positive control (challenged with toxigenic *C. difficile* strain), GROUP 5 animals received the probiotic non-toxigenic *C. difficile* strain and then challenged with the toxigenic *C. difficile* strain, GROUP 6 received probiotic yogurt and then challenge with the toxigenic *C. difficile* strain. Piglets were individually housed with non-challenged piglets (Groups 1 -3) being house in separate airspace than challenged piglets (Groups 4-6). Piglets were administered the probiotic 4 hours post birth and challenged 16 hours later with a 4+ toxin producing filed isolate and euthanized 72 hours post-challenge. At necropsy, gross observations at necropsy included 1) body condition, 2) dehydration status, 3) perineal fecal staining, 5) consistency of colonic contents, 6) mesocolonic edema, and the presence of 7) visible colonic luminal necrosis. Fresh and formalin fixed tissues including ileum, jejunum, descending colon, cecum, and a cross section of spiral colon were collected for culture and histopathologic examination. Rectal swabs collected prior to inoculation and pooled colon and cecum contents collected at necropsy were assayed for *C. difficile* toxins using a commercially available toxin ELISA. For statistical analysis, three categories of scores were compared: 1) clinical signs, 2) ELISA results and mesocolonic edema, and 3) microscopic lesions. Clinical signs scores were created by summing scores for body condition, hydration status, and perineum staining. Overall statistical evaluation of clinical signs scores from study groups revealed no statistical difference ($P>0.05$). Group 4 presented significant higher scores when compared to Groups 1, 2 and 5 ($P=$ 0.04, 0.018 and 0.002 respectively. Histopathologic examination revealed classic CDI microscopic lesions characterized by variable numbers of intestinal only observed within colon and cecum. Group 4 had significant higher microscopic scores when compared to Groups 2 (P=0.005) and 5 (P=0.02). Analysis of data indicated that mesocolonic edema, ELISA toxin levels

and histologic lesions are highly and significant correlated (*P*=0.001). Under our experimental conditions, the use of non-toxigenic *C. difficile* probiotic was effective in minimizing the histologic lesions associated with *C. difficile* infection.

Introduction:

In the last 10 years, *Clostridium difficile* has been implicated as a major cause of neonatal diarrhea in pigs. *C. difficile* infection (CDI) affects piglets ranging in age from 1-7 days. Clinical signs of CDI include diarrhea, abdominal distention and scrotal edema with most of the pathology being attributed to toxins A (TcdA) and B (TcdB). The prevalence of *C. difficile* is widespread and it has been referred to as the most important uncontrolled cause of neonatal diarrhea in the pig. This is supported by many studies indicating a prevalence rate of about 50% and the fact that *C. difficile* may affect litter productivity by as much as 10-15%. Currently there are no commercial vaccines against *C. difficile* and the use of prophylactic antibiotics has been unsatisfactory and unrewarding for swine producers.

Clostridium difficile infection is currently considered one of the most common causes of nosocomial diarrhea in humans. In humans, the use of antimicrobials and the subsequent disruption of intestinal flora is an important predisposing factor in the development of CDI. It has also been shown that probiotics are a beneficial addition to conventional therapy of CDI. Therefore, we hypothesize that early oral administration of probiotics to pigs will control disease after experimental infection with *C. difficile*.

The work outlined in this proposal could lead to an economical, safe, and simplistic therapy to control CDI and its subclinical incidence. By using competitive exclusion we will be negating the use of antibiotics, either in prophylactic application or treatment. With antibiotic-resistance becoming an issue for both human and animal health, effective alternatives need to be developed and investigated. This also has major implications for this disease of increasing importance in human medicine. CDI is one of the most common nosocomial infections in the elderly and accounts for 25% of the antibiotic induced diarrheas in human adults (Keel et al., 2006; Songer and Anderson 2006).

Objectives:

Central hypothesis**:** Oral administration of probiotics to newborn pigs will control disease after experimental infection with *C. difficile*. Our long-term research goal is to gain a better understanding of *C. difficile* and the porcine intestinal microbiota and how it factors into disease. In this study, we will investigate if early intestinal colonization can competitively exclude *C. difficile* and prevent disease.

Objective 1: Demonstrate that probiotics are capable of controlling clinical signs associated with *C. difficile* infection.

Objective 2: Demonstrate that probiotics are capable of limiting lesions consistent with *C. difficile* infection.

Materials & Methods:

Animals

Pregnant commercial cross-bred sows were purchased and delivered to Iowa State University (ISU) approximately one week prior to expected farrowing date. On day 113 of gestation, caesarian surgeries were performed on sows for piglet derivation. All one hundred and fifty five piglets included in this study were triaged at birth with navels clamped, cut, and sprayed with gentile iodine. Piglets also received an iron injection. Piglets were kept BSL-2 animal facility at ISU. Sera from all procured neonatal pigs were negative for porcine reproductive and respiratory syndrome virus (PRRSv) nucleic acid by PCR using a licensed, commercially available real time PCR assay (Applied Biosystems, CA, USA).

Housing

Piglets were individually housed in brand new 18 gallon plastic totes (Rubbermaid®, Port Washington, NY). All challenged pigs were housed in the same room and airspace. Negative control piglets were housed in a separate room and airspace. Piglets housing and daily care have been described by Arruda et al (2008). In summary Piglets were fed milk replacer (Esbilac; Pet-Ag, Hampshire, IL) three times a day (7 am, 12 pm, and 7 pm). All feedings were done by oral-gastric intubation using an 8 gauge French catheter (Sovereign TM, Tyco/Healthcare, Mansfield, MA).

Experimental Design

Study design contained six separate groups as follow: GROUP 1 negative control, GROUP 2 piglets only received non-toxigenic *C. difficile* strain, GROPU 3 piglets received only probiotic yogurt, GROUP 4 positive control (challenged with toxigenic *C. difficile* strain), GROUP 5 animals received the probiotic non-toxigenic *C. difficile* strain and then challenged with the toxigenic *C. difficile* strain, GROUP 6 received probiotic yogurt and then challenge with the toxigenic *C. difficile* strain. Table 1 summarizes the study design. Four replicates of the study were performed, summing up to 150 total animals. In each experimental replicate, pigs were randomly allocated into one of the six groups using several random number iterations in Microsoft Excel®. The experimental protocol was approved by the ISU Institutional Animal Care and Use Committee.

Probiotic treatments were administrated, according to experimental design, four hours after delivery. Piglets from GROUP 2 and 5 received $2x10^6$ heat-shocked non-toxigenic *Clostridium difficile* spores and piglets from GROUP 3 and 6 received yogurt with $2x10^6$ *Lactobacillus spp*. Sixteen hours following probiotic administration, groups 4, 5, and 6 were challenged with $2x10^6$ heat-shocked toxigenic *Clostridium difficile* spores (Table 1). All piglets were euthanized 72 h post challenge.

Table 1. Experimental design

Group	n	Treatment	Probiotic	Inoculation	Dose
1	10	Control	-	No	-
2	13	Probiotic 1	Non-toxigenic *C. difficile* spores @ 2 $x10^6$	No	-
3	14	Probiotic 2	*Lactobacillus spp*. @ 2 $x10^6$ in yogurt	No	-
4	35	Placebo	-	Yes	Toxigenic *C. difficile* spores @ 2 $x10^6$
5	34	Probiotic 1	Non-toxigenic *C. difficile* spores @ 2 $x10^6$	Yes	Toxigenic *C. difficile* spores @ 2 $x10^6$
6	44	Probiotic 2	*Lactobacillus spp*. @ 2 $x10^6$ in yogurt	Yes	Toxigenic *C. difficile* spores @ 2 $x10^{66}$

Inoculum

Toxigenic *C. difficile* isolate ISU-15454-1, was utilized for all replicates. This isolate originated from a field case of neonatal diarrhea in 3-6 day-old piglets with high levels of toxin (4+) detected by ELISA (C. DIFFICILE TOX A/B IITM, Balcksburg, VA). Isolate 15454-1 is ribotype 078, toxinotype V, and contains both toxin A and toxin B gene sequences (Yaeger et al., 2007). The isolate was stored at -80°C.

Procedures involving *C. difficile* isolation, growth and harvest of spores has been previously described (Lizer et al., 2013). Furthermore, spores titration and heat shock activation prior challenge was done as described by Lizer et al., 2013.

Inoculation

All piglets were intragastrically inoculated using an eight-gauge rubber French catheter as an oral-gastric tube (Sovereign TM, Tyco/Healthcare, Mansfield, MA). Probiotic inoculation occurred approximately four hours after delivery. A 1.25 ml of probiotic preparation containing heat-shocked non-toxigenic *C. difficile* spores or 5 ml of 2 X 10^6 *Lactobacillus spp* in yogurt were given followed by 20 ml of milk replacement. Sixteen hours later, 1.25 ml of inoculum preparation containing heat-shocked *C. difficile* were administrated to piglets according to Table 1.

Lactobacillus spp Probiotic

Ultra 75 Billion CFUs Probiotic Complex (GNC, General Nutrition Corporation, Pittsburg, PA) was used to fortify amount of *Lactobacillus acidophilus in yogurt*. On capsule containing 75 billion CFUs of probiotic was thoroughly mixed with 190 ml of yogurt. Each piglet was administered 5 ml of this fortified yogurt to provide 2 x 10^6 *Lactobacillus spp*.

Necropsy

Piglets were monitored for 72 h post-challenge and then euthanized by an intravenous overdose of pentobarbital. Gross observations at necropsy included 1) body condition, 2) dehydration status, 3) perineal fecal staining, 5) consistency of colonic contents, 6) mesocolonic edema, and the presence of 7) visible colonic luminal necrosis and were scored independently in a blinded fashion as previously described (Yager et al., 2007, Lizer et al., 2013, Arruda et al., 2013). Necropsies, clinical sign scores and gross lesion scores were completed by the same two individuals for all experiments.

Sample collection

Rectal swabs were taken from all piglets prior to inoculation. At necropsy, fresh and formalin fixed tissues were collected and instruments were properly disinfected between animal necropsies. Samples included: ileum, jejunum, descending colon, cecum, and a cross section of spiral colon containing 4-5 loops. Colonic and cecal contents were collected and storage in sterile plastic cup. Ileum swab (Dacron® Fiber Tipped, Fisher brand®, Leicestershire, UK) was also taken at necropsy.

Toxin detection and culture

Rectal swabs collected prior to inoculation and pooled colon and cecum contents collected at necropsy were assayed for *C. difficile* toxins. A commercially available toxin ELISA kit (C. DIFFICILE TOX A/B II™, Blacksburg, VA) was used to semi quantitatively measure the amounts of toxin from 0 (no toxin detection) to 4+ (marked toxin detection) as indicated by the manufacturer. Samples previously described were frozen and storage at -80°C until completion of study. All samples were tested at the same time and in accordance with manufacturer instructions analyzed on a microplate reader (IDEXX Corp, Molecular Device, Lake Forest, IL).

Pooled large intestinal contents and mucosal scrapings of fresh spiral colon were combined and cultured on *C. difficile* selective agar (CDSA) (Stool-Prep, TechLab, Blacksburg, VA); both direct and following a 30 minute room temperature incubation in 0.5 ml absolute ethanol. All plates were incubated at 37°C for 48 h in an anaerobic chamber. *Clostridium difficile* growth following incubation was semi-quantitatively scored in a blinded manner by a veterinary microbiologist as follow: 0 = no growth, 1 = few colonies, 2 = low numbers of colonies, 3 = moderate growth and 4= high growth. Rectal swabs collected prior to inoculation from all piglets were also cultured as described above. Ileum swabs collected at necropsy were tested by routine aerobic and anaerobic culture methods for *Salmonella* spp, *Escherichia coli*, and *Clostridium perfringens*. Genotyping for *E. coli*, and *C. perfringens* were performed (Casey and Bosworth, 2009; Meer and Songer, 1997) to determine surface antigen and associated toxin genes.

Histopathology

Tissue sections were collected in 10% neutral buffered formalin. Sections were submitted to routine tissue sectioning followed by paraffin embedding and staining with hematoxylin and eosin. All tissues were examined by a veterinary pathologist that was blinded to animal group designation. Large intestinal sections were assessed for goblet cell loss, neutrophilic aggregates within the lamina propria, and mucosal epithelial defects as previously described (Lizer et al., 2013; Arruda et al., 2013).

Scoring

Three categories of scores were compared: 1) clinical signs, 2) ELISA results mesocolonic edema, and 3) microscopic lesions. Clinical signs scores were created by summing scores for body condition, hydration status, and perineum staining. Mesocolonic edema was score by blind pathologists at necropsy. Microscopic lesion score was the sum of scores for all histopathology categories as previously described (Lizer et al., 2013; Arruda et al., 2013).

Statistical methods

Scores for clinical signs, gross and microscopic lesions were analyzed by a non-parametric test. Wilcoxon/Kruskal-Wallis test was used to determine if differences existed between study groups, and isolate groups to each other. Correlations between histologic lesions, mesocolonic edema and ELISA results were accessed by Spearman's rank correlation coefficient; a non-parametric test. JMP 9 (JMP®, Cary, NC) statistical software was used to perform analyses.

Results:

Objective 1 (Clinical signs)

Clinical scores were independently and blindly scored for all pigs at completion of study. Overall statistical evaluation of clinical signs scores from study groups revealed no statistical difference ($P>0.05$). However pair comparisons indicated the majority of piglets at necropsy from Groups 1, 2 and 3 presented with normal body condition as well as hydration status; 90% (9/10), 85% (11/13) and 93% (13/14) respectively. A similar trend was observed with challenge groups (Groups 4, 5 and 6) however there is a numerical difference between groups that were inoculated with the toxigenic *C. difficile* strain and the first three control groups. Piglets presented with normal body condition as well as hydration status accounted for only 66% (23/35), 61% (21/34), and 61% (27/44) respectively.

Objective 2 (Gross and Histologic lesions)

Gross lesions

Statistical analysis revealed an overall significant difference in mesocolonic edema scores ($P=0.01$). Pairwise comparison was performed and revealed the following results. Group 4 presented significant higher scores when compared to Groups 1, 2 and 5 ($P=$ 0.04, 0.018 and 0.002 respectively). Other pair comparisons failed to achieve statistical significance. Grossly visible mucosal necrosis was not seen within the cecum or spiral colon of any piglet. All animals were ELISA negative at the beginning of the experiment. At necropsy (72 hours post inoculation), Group 5 had significant lower levels of toxin A and B detected via ELISA in colonic content when compared to Groups 3, 4 and 6.

Microscopic lesions

Histopathologic examination revealed classic CDI microscopic lesions characterized by variable numbers of neutrophils within lamina propria, a loss of goblet cells and single to multiple sites of erosions and ulcerations which occasionally are covered by moderate amounts of cellular and karyorrhectic debris and fibrin; lesions were only observed within colon and cecum. Microscopic scores were statistically different between groups ($P=0.03$). Statistical comparison among groups was performed and results revealed that Group 4 had significant higher scores when compared to Groups 2 ($P=0.005$) and 5 ($P=0.02$). Also Group 2 had statistically lower scores than group 1 ($P=0.02$). No other pairwise comparisons were significantly different (Table 3).

Table 2. Histologic scores

Group	Mean (histologic score)*	SEM¶
1	0.80	0.32
2	0.07	0.07
3	1.92	1.02
4	3.54	0.81
5	1.05	0.52
6	1.84	0.56

*See Materials and Methods for details of histologic scores

¶ SEM = Standard error of the mean

Table 3. Pair comparison of histologic scores and respective (*P*) values (Wilcoxon method)

Group comparison	Score mean difference	Std. error Diff ¶	(*P*) value
1 X 2	-4.95	2.19	**0.02***
1 X 3	-2.05	2.45	0.40
1 X 4	3.79	4.40	0.38
1 X 5	-3.94	3.88	0.30
1 X 6	-2.94	4.61	0.52
2 X 3	2.00	1.88	0.28
2 X 4	11.1	4.00	**0.005***
2 X 5	5.31	3.31	0.10
2 X 6	6.67	3.95	0.09
3 X 4	5.95	4.06	0.14
3 X 5	0.65	3.47	0.85
3 X 6	1.60	4.07	0.69
4 X 5	-9.6	4.28	**0.02***
4 X 6	-8.9	4.57	0.05
5 X 6	1.35	4.16	0.74

*Indicate statistical significance with (*P*) value < 0.05
¶ Indicate Standard error of the differences

Associations
Associations among mesocolonic edema, toxin levels and histopathologic lesions were accessed by Spearman`s rank correlation coefficient. Analysis of data indicated that mesocolonic edema, ELISA toxin levels and histologic lesions are highly and significant correlated (*P*=0.001 on all combinations).

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

Our experiments were successful in challenging our piglets as noted in gross and histologic scores. This project did demonstrate that the use of non-toxigenic *C. difficile* probiotic was effective in minimizing the histologic lesions associated with *C. difficile* infection. This is most likely due to competitive exclusion although our study was not designed to determine this. Interestingly this decrease was also seen in the non-challenged piglets that received this non-toxigenic probiotic (*P*=0.02). This is a very interesting finding which suggests they be some other "healthy" effects of having non-toxigenic *C. difficile* bacteria in the cecum and spiral colon; a true probiotic effect. This finding also brings up the question on even how more effective a probiotic non-toxigenic *C. difficile* can be when considering it is likely there are some low level and subclinical infections.

The *Lactobacillus spp* probiotic also appeared to trend (*P*≤0.10) to have slightly lower histologic lesions when comparing to Groups 5 and 6. It could be that for the *Lactobacillus spp* probiotic a continuous daily dose may be necessary to provide effective protection against CDI.

It was also very interesting that for the first time, to our knowledge, we were able to see a direct association between mesocolonic edema, toxin levels and histologic lesions. This supports our current understanding of CDI as a toxin mediated disease. It is very feasible that non-toxigenic *C. difficile* probiotic can be easily used in the field to mitigate CDI in newborn piglets.

Some of the limitations on this study include the fact that we provided the probiotics as a single large dose and had full control over the timing of infection. We did ensure piglets would become infected 16 hours post probiotic

administration and used a high dose challenge. We also only evaluated effects at 72 hours not allowing us to gain better insight on how fast the probiotics would work. By having 150 piglets in this study, we were able to see some statistical differences between groups. The high number of animals was needed due to the normal variation in intestinal lesions seen in piglets of this age. The cause for some for those lesions was not fully explored in his study.

Looking into the future, it appears to be very feasible and practical that a non-toxigenic *C. difficile* probiotic could be used on farm to improve overall gut health and mitigate intestinal damage due to CDI.