RESEARCH REPORT
SWINE HEALTH

Title: Testing of a live enterotoxigenic E. coli vaccine candidate for its potential as a competitive exclusion probiotic for preventing colibacillosis in weaned pigs – **NPB #08-077**

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Industry Summary

The bacterium Enterotoxigenic Escherichia coli (E. coli) is a major cause of post-weaning scours in weaned pigs, most frequently causing disease shortly after weaning. Currently no licensed vaccines are available and treatment options are limited, sometimes expensive and marginally effective. We sought an inexpensive and effective preventive measure easily applied to prevent this post-weaning disease in pigs. We hypothesized that an experimental live E. coli vaccine under development in our laboratory may have value as a probiotic for pigs. To test that hypothesis, we fed the vaccine in either of three forms to five-day-old pigs, which should be even more susceptible to E. coli than weaned pigs. Twenty-four hours after piglets were given the vaccine strains, they were challenge-inoculated with highly virulent enterotoxigenic E. coli. The experiment was terminated 24 hours after the challenge and the condition of the pigs was assessed. We found that pigs receiving the complete vaccine were highly protected from disease. They did not develop scours or exhibit other signs of illness. Further, upon postmortem examination, we found that their intestines were largely spared infection by the pathogen. Such as not the case with pigs not given the vaccine strain. They became very ill and their intestines were highly infected. In further studies, we found that the vaccine strain needed to posses the key traits at enabled it to colonize the intestines to be effective. Further, it had to be able to grow rapidly in the intestines and had to possess similar traits to the pathogen to be effective. It appears that the vaccine strain acts as a probiotic by arriving in the intestines before the pathogen and there colonizing the tissues, competitively excluding the pathogen which then finds no place to colonize. We conclude that this approach to a probiotic would provide a convenient and highly protective approach to preventing post-weaning scours during the first days after weaning. Other studies in our laboratory indicate, that piglets do develop immunity from the vaccine strain, which terns the short-term probiotic protection into long-term immunity. Thus, pigs would be perminantly protected from E. coli when this vaccine strain is utilized.

Scientific Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in both humans and livestock. Virulence of ETEC is associated with fimbrial adhesins that enable attachment to the mucosa of the small intestine and enterotoxins such as heat-labile (LT) and/or heat-stable (ST) enterotoxins that stimulate host diarrhea. Our recent *in vitro* work using a cell culture model system suggests that exclusion of ETEC from attachment to epithelial cells requires expression of both an adhesin such as K88 fimbriae, and the LT enterotoxin. In addition, we have observed that LT contributes to ETEC adherence independent of secretory activity. To further test the ability of non-pathogenic *E. coli* (probiotic) strains to colonize the intestine and competitively exclude ETEC at the level required to produce clinical disease, we utilized a piglet ETEC challenge model. Thirty-nine 5-day-old piglets were inoculated with either a placebo (negative control group) or with any of the three $K88^+$ *E. coli* strains isogenic with regard to modified LT expression. The isogenic strains were 8017 (pBR322 control), the non-toxigenic mutant 8221 (LT(R192G) in pBR322), or 8488 (LT(R192G) gene fused to the gene for STb in pBR322). Piglets were challenged with virulent ETEC strain 3030-2 ($K88^+$/LT/STb) 24h after piglets were inoculated with the isogenic strains. All K88ac receptor-positive piglets in the control group (given no isogenic strain) developed diarrhea and became dehydrated after 12 hours post-challenge. Piglets inoculated with strains 8221 or 8488 did not exhibit any clinical signs of ETEC disease while piglets inoculated with ETEC strain 8017 showed mild diarrhea to no diarrhea post-challenge. There was significant weight loss in the control pigs compared to the piglets inoculated with the isogenic strains and blood total protein was significantly higher in the control pigs compared to the pigs inoculated with strains 8221 or 8488. Quantitative culture of the challenge strain in washed ileum and jejunum indicated a significant higher number of pathogenic *E. coli* in the control group compared to groups inoculated with strains 8017 or 8488 for both segments of the small intestine. There was a significantly higher number of pathogenic *E. coli* in the ileum of pigs inoculated with strain 8017 compared to the 8488 group. In further studies to assess whether the protection was highly specific to K88 ETEC, we inoculated the piglets with the strains competitive exclusion strains as indicated above, but challenged with an unrelated ETEC strain (B44; O9:K99:F41: H-; STa). The vaccine strains were not protective against this unrelated ETEC, suggesting that protection is highly specific. In further studies, we substituted the vaccine strains with strains K12 strains possessing K88 fimbriae and the modified LT toxin. K12 strains grow poorly if at all in mammalian intestines. Thus, these bacteria are living, but non-proliferative. When pigs were treated with these strains, then challenged with K88+ ETEC, they were not protected. This observation would suggest that protection may require sufficient bacterial proliferation for substantial colonization to occur. Thus, it is unlikely that the mechanism of protection is primarily an up-regulation of innate immunity precipitated by epithelial contact with a few ETEC-like bacteria. Rather it is likely a competitive exclusion event made possible by a large population of bacteria similar the ETEC pathogen, but unable to cause diarrheal disease.

Project Objective: To establish whether piglets receiving the candidate probiotic/vaccine strain, then challenged with ETEC 24h later are protected from the challenge strain.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of illness and death among recently weaned pigs (Nagy & Fekete 1999, 2005). ETEC has caused major economic losses in the farming industry due to increased mortality, reduced production efficiency and increased treatment costs (Fairbrother et al., 2005). ETEC strains expressing K88 fimbriae and heat-labile enterotoxin (LT) and heat-stable enterotoxin b (STb) account for the majority of the ETEC strains isolated from clinically ill pigs. In addition, all LT^+ strains among swine isolates in the United States are of serotypes

expressing the K88 fimbriae (Wilson & Francis, 1986; Francis, 2002; Freydahl, 2002; Zhang et al., 2006).

Susceptibility of piglets to $K88^+$ ETEC strains is genetically determined and inherited as a dominant trait. In a survey of purebred pigs, Baker et al. (1997) found that about 40% of the population they examined exhibited a phenotype correlated with susceptibility to $K88^+$ ETEC. Susceptibility of pigs to $K88^+$ ETEC is due to the expression of a high molecular mass mucin-type glycoprotein (IMTGP) identified on enterocyte brush borders (Erickson et al., 1992, 1994). Animals that do not express the glycoprotein receptor are not susceptible to the disease.

Early piglet weaning programs (approximately 3 weeks of age) and piglet vulnerability to ETEC infection immediately after weaning leaves little time for employment of a vaccine program, even if a vaccine were available. The newborn piglet has an immature immune system (Butler et al., 2002), and maternal antibodies may interfere with the response to an ETEC vaccine. At present, ETEC infection of suckling pigs can be prevented through maternal immunization and by early supply of immune colostrum. Although highly effective vaccines have been developed for the neonatal form of the disease (Moon & Bunn, 1993), little progress has been made for preventing the disease in older animals mainly due to the difficulty in stimulating mucosal immunity using non-living antigens. One possible solution to the ETEC disease problem in weaned pigs is using a competitive exclusion product that may also function as a live vaccine. If effective, such an organism would colonize the intestine and competitively exclude ETEC at the level required to produce clinical disease.

Our recent *in vitro* work suggests that exclusion of ETEC from attachment to epithelial cells requires expression of both an adhesin such as K88, and an enterotoxin such as LT. Therefore, the goal of this study was to test a vaccine candidate *in vivo*, to establish proof of the probiotic principle in preventing ETEC disease.

Materials and Methods

Bacterial strains and plasmids. Bacterial **s**trains used in this study are described in Table 1. The parental strain used to construct the isogenic probiotic strains was 1836-2, a non-pathogenic O8:H4 porcine *E. coli* field isolate which expresses K88ac fimbriae but no known enterotoxins. Isogenic strain 8017 (control) was constructed by transforming 1836-2 with pBR322 (Zhang et al., 2006). Isogenic strains 8221 and 8488 were constructed by transforming 1836-2 with pBR322 containing LT(R192G) and LT(R192G) plus STb, respectively. The LT(R192G) was constructed by site-directed mutagenesis as described previously (Dickinson and Clements). Strain 3030-2, is an O157 wild-type ETEC strain that expresses K88 fimbriae, LT and STb. Strain B44 is an O9 wild-type ETEC strain that expresses K99 and F41 fimbriae and STb enterotoxin. Strain TK88-1 was created by conjugation to transfer the K88ac adhesin encoding plasmid and the LT enterotoxin encoding plasmid of the 1477 wild-type ETEC strain into a spontaneous nalidixic acid resistant mutant of the C600 (K12 E. coli) library strain.

Animals. Piglets derived by natural farrowing and allowed to suckle colostrum from the sow for 12 hours after birth. After 12 hours of birth, piglets were separated and reared in individual cages and each litter was divided randomly into two to four treatment groups, depending on litter size and experimental design. Experiments were preapproved by the South Dakota State University Institutional Animal Care and Use Committee.

Piglet brush border adherence assay for the assessment of K88 receptor expression. Jejunum samples collected from each piglet at necropsy were used to prepare brush border vesicle as described by Baker et al. (1997). The phenotype of brush border vesicles was tested for *E. coli* expressing K88ab, K88ac, and K88ad fimbriae using the method described by Sellwood et al (1975) and modified by Bahler et al. (1997). In a 20-well seroagglutination plate, suspensions of purified

brush borders (50 µl) from each piglet were mixed with 10% D-mannose (50 µl) to block mannose-sensitive adherence. Bacterial suspensions (25 µl) were then added, and mixtures were incubated for 5 min on an orbital shaker at room temperature. Bacterial adhesions to brush border vesicles were observed under phase microscopy at 40X total magnification. The percentage of adhesive brush borders was determined by counting at least 20 brush borders. Brush border specimens containing at least 10% adhesive brush borders were considered positive for adherence. Individual brush borders were considered adherent if there were more than two bacteria adhering to a brush border vesicle.

Oral inoculation of piglets. Piglets were fed Esbilac milk replacer (Pet Ag, Inc) after weaning from the sow. At 5 days of age, each piglet was fed 3 ml of an overnight culture of the test *E. coli* vaccine strain to be used as a probiotic grown in Luria-Bertani (LB) broth (~ 3 x 10^9 CFU) mixed with milk replacer. Control groups were given only 3 ml LB broth in milk replacer. After 24 hours of feeding the probiotic strains, the piglets were challenged with ETEC strain 3030-2 (O157: K88/LT/STb) or in one case B44 (O9:K99/F41/STa). Each piglet was fed 3 ml of an overnight culture of the challenge strain mixed with milk replacer. Piglets were monitored for the following clinical signs of ETEC disease: anorexia, vomiting, diarrhea, depression, and moribund condition. Piglets were observed for the above parameters every 4 h until 24 h post-challenge or until they become moribund which ever occurred first, at which time they were subjected to euthanasia and necropsy. A moribund condition was defined as previously described by Berberov et al. (2004) — severe dehydration (evidenced by loss of skin turgidity and eyes sunken into the orbits), severe weight loss (>15%), depression, hypothermia, and severe weakness. At euthanasia, samples of the ileum (3-5 cm proximal to the ileocecal valve), lower jejunum (one-third to one-half of the distance between the pyloric valve and the ileocecal valve), upper jejunum (one-half to two-thirds of the distance between the pyloric valve and the ileocecal valve), and duodenum (3 to 5 cm distal to the pyloric valve) were collected for bacteriology and histopathology studies.

Experimental Design:

Experiment 1. Assessment of protection mediated by homologous probiotic strains. A total of 31 piglets were treated with vaccine (probiotic) strain 8017, 8221, or 8488, or no probiotic, 9,8,7 and 7 piglets respectively) then challenged with ETEC Strain 3030-2.

Experiment 2. Assessment of protection mediated by heterologous probiotic strains. Three piglets were treated with the same vaccine strain 8488 then challenged with ETEC strain B44. Three pigs served as controls (not treated with probiotic, but challenged with Strain B44.

Experiment 3. Assessment of protection mediated by a homologous living, but a non-proliferating probiotic strain. In the principal group 5 piglets were treated with the TK88-1 (K88ac adhesion, and LT enterotoxin producing) strain. The 4 piglets of the control group were treated with the “empty” (virulence determinante-negative) C600N laboratory strain. Twenty-four h later, piglets were challenged with ETEC Strain 3030-2.

Assessment of piglet dehydration. The level of dehydration following challenge was determined by measuring changes in weight, blood packed cell volume (PCV) and plasma total protein (TP). Weight and blood samples were taken from each piglet prior to challenge and upon euthanasia. Blood was tested for PCV and TP as described by McCrunin (1998). Briefly, each blood sample was placed in a 75-mm capillary tube and centrifuged for 5 min for PCV and TP analysis. PCV was determined using a standard hematocrit percentage chart. Plasma TP content was determined using a standard refractometer. Weight loss, increase in PCV and plasma TP from pre-challenge sample collection to necropsy sample collection served as an indication of dehydration.

Assessment of bacterial intestinal colonization. Determination of the magnitude and location of bacterial colonization of the small intestine was accomplished by quantitative culture and analysis of histopathology sections of the piglet small intestine. The concentration of bacteria in colony-forming units (CFU) per gram of small intestinal tissue was determined as previously described by Francis et al. (1998). Briefly, intestinal tissue was weighed, washed, and ground in PBS (at a ratio of N grams of tissue in 9 x N milliliters of PBS), serially diluted, plated on blood agar or LB plates, and incubated at 37°C, after which hemolytic bacterial colonies (ETEC strain 3030-2) were enumerated.

Tissue samples from the duodenum, upper jejunum, lower jejunum, and ileum of each challenged piglet were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, mounted on glass slides, and stained with Giemsa by standard staining procedures. Giemsa-stained tissue sections were examined by light microscopy and scored blindly on a 5-point scale using the method of Bertschinger et al. (1972). Areas on the villi where bacteria were found in proximity to the epithelium were identified, and the tendency of these bacteria to be contiguous to the epithelial cells was scored from 1 for no bacteria to 5 for the maximum number of bacteria, based on the following criteria: 5 = bacterial adherence is confluent from the base to the tip of the villi; 4 = bacterial adherence in confluent along lateral edge of the villi but lacking at the base or tip; 3 = bacterial adherence is non-confluent and localized to small patches of colonies; 2 = 5 bacteria or less per colony associated with epithelium; and 1 = lack of adherent bacteria or bacterial association with villi. Twenty villi from each paraffin section were scored in the criteria described above. Tissue sections were also stained by standard immunohistochemistry procedures as described by Rogers et al. (1996) using rabbit polyclonal antiserum raised against E. coli O157 somatic antigen (Denka Seiken USA, Campbell, CA) as the primary antibody, goat anti-rabbit IgG (heavy and light chain specific), avidin-biotin alkaline phosphatase solution, and Vector Red as the substrate (Vector Laboratories). Microscopic images were examined to verify if the attached bacteria were ETEC strain 3030-2.

Statistical analysis. Student's *t* test was used to compare the means of the different treatment groups. Calculated *P* values of <0.05 were considered significant.

Results

Phenotypic analysis of piglets used in this study. The success of this study specifically depended on experimental pigs expressing the 210 and 240 kDa IMTGP receptor. Enterocytes from phenotype A and B piglets express the IMTGP receptor and also support adherence of K88ac fimbriae. Presence of IMTGP receptor was validated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot techniques (data not shown). Data from approximately 10% of piglets were eliminated from this study because they failed to exhibit K88 receptors.

Results from Experiment 1.

Oral inoculation with attenuated LT expressed by K88ac$^+$ E. coli prevented dehydrating diarrhea in K88-receptor positive piglets challenged with wild-type ETEC. Nine out of 13 piglets belonging to the control group (pre-inoculated with LB broth only) were positive for the K88 receptor, IMTGP. All 9 piglets exhibited watery diarrhea, dehydration and anorexia after oral challenge with wild-type ETEC strain 3030-2. Clinical responses of piglets given ETEC strain 8017 were variable, ranging from no diarrhea to moderate diarrhea although all 8 piglets were K88ac receptor positive. On the other hand, 1 out of 8 piglets fed ETEC strain 8221 and 3 out of 10 piglets fed ETEC strain 8488 were negative for the presence of IMTGP. However, no piglets in either group showed any clinical signs of ETEC infection. Upon necropsy, we observed that piglets that exhibited diarrhea had hyperemic intestines and gas and/or fluid accumulation in the small and/or large intestines.

The mean weight loss in the control group was significantly higher compared to treated groups ($P < 0.001$; Figure 1). Increases in PCV and TP were detected in piglets that became dehydrated as a result of challenge with strain 3030-2. TP values of piglets in the control group were statistically higher compared to piglets fed 8221 and 8488 ($P < 0.001$); however, there was no statistical difference in the PCV of the control versus treated pigs (Figures 2 and 3).

Evaluation of in vivo adherence by colony counts and microscopy. Quantitative culture of ETEC strain 3030-2 present in the washed ileum indicated a significant higher concentration of adherent bacteria (CFU/g washed ileum) in control piglets compared with those inoculated with strains 8017 and 8488, and piglets inoculated with strain 8488 had significantly higher bacterial adherence compared with those inoculated with strain 8017 (Figure 4). There was an increase of almost 2 logarithmic values in the concentration of adherent bacteria in piglets belonging to the control group (1.4×10^9) compared with piglets inoculated with strain 8488 (8.2×10^7; $P = 0.002$). Piglets inoculated with strain 8017 had at least ½ log less adherent bacteria in the ileum (4.6×10^8) than the control pigs ($P = 0.05$). In addition, piglets inoculated with strain 8017 had a significantly higher concentration of adherent strain 3030-2 compared with piglets inoculated with strain 8488 ($P = 0.05$). Although there was almost 1 log difference in the concentration of adherent bacteria between the control piglets those inoculated with strain 8221 (1.4×10^9 versus 2.5×10^8, respectively), there was no significant difference due to the high variability in the concentration of bacterial adherence in the ileum of piglets inoculated with strain 8221 ($P = 0.36$; Figure 4).

Likewise, quantitative culture of ETEC strain 3030-2 present in the washed jejunum indicated a significant higher concentration of adherent bacteria (CFU/g washed jejunum) in control piglets compared with those inoculated with strains 8017 and 8488 (2.1×10^9, 3.7×10^8, and 2.3×10^8, respectively; $P < 0.05$; Figure 5). There was almost 1 log difference in the adherence of bacteria between the control group and group inoculated with strain 8221; however, the difference was not statistically significant due to high variability in the concentration of bacterial adherence in the jejunum of piglets inoculated with strain 8221 ($P = 0.84$; Figure 5).

Sometimes, viable bacteria counts taken after washing of intestinal sections are not indicative of what is seen through the microscope. Therefore, scoring the Giemsa-stained paraffin sections on the basis of bacterial association and examining the immunohistochemistry slides was employed to validate adherence of ETEC strain 3030-2.

Using the 5-point scale method of Bertschinger et al. (1972), there was highly significant number of bacteria adhered to the ileum and jejunum of the control piglets compared to those inoculated with 8221 and 8017 ($P = 0.002$ and 0.01, respectively; Tables 3a and 3b). Examination of immunohistochemistry slides made from the same tissue sections which were used for Giemsa-stained slides revealed that while strain 3030-2 was attached in high concentration on the intestinal epithelial surface of the control pigs, such is not the case in the pigs inoculated with the isogenic strains (Figure 6). One possible explanation why we did not find any significant difference in bacterial attachment to the intestinal epithelial surface of the control pigs versus pigs inoculated with 8488 was that control pigs had a high concentration of the challenge strain attached to their intestinal epithelial surface while pigs inoculated with 8488 had a high concentration of 8488 attached to their intestinal epithelial surface, and Giemsa does not differentiate between the 2 strains. Therefore, it is necessary to examine immunohistochemistry slides to validate what strain is attached to the intestinal epithelial surface of the experimental pigs.

Results from Experiment 2.

Piglets inoculated with the K88/LT mutant vaccine strain were not protected when challenged with Strain B44 which is an unrelated to the vaccine strain in that its fimbriae are K99 and F41, and its toxin is STa.

Results from Experiment 3.
Piglets inoculated with TK88-1 (the K88/LT-expressing K12, non-proliferating substitute of the vaccine strain) were not protected upon challenge with ETEC Strain 3030-2.

Discussion

This study was performed to establish the ability of constructed isogenic ETEC strains to competitively exclude virulent ETEC. Using an ETEC piglet challenge model, 5-day old piglets were inoculated with an oral ETEC isogenic construct or placebo, followed by a highly virulent ETEC challenge strain 24 hours later. Isogenic constructs were tested whether K88 expression alone, or accompanied by attenuated LT or attenuated LT/STb provided protection from the effects of the pathogenic ETEC strain. They were further tested to determine whether the constructs could protect against an ETEC with unrelated virulence determinants (K99/F41/STa) or whether proliferating strains were required to provide competitive exclusion. We found that both K88 fimbriae and an LT derivative (LTR192G) were important to the provision of clinically protective competitive exclusion. We found further, that competitive exclusion was virulence-determinant specific, in that that cross protection across fimbrial types did not occur. It was additionally noted that non-proliferating bacteria (K12 organisms) were unable to provide competitive exclusion protection. K12 strains grow poorly, if at all in mammalian intestines. Thus, these bacteria are living, but non-proliferative. This lack of protection by K12 strains would suggest that protection may require sufficient bacterial proliferation for substantial colonization to occur. Thus, it is unlikely that the mechanism of protection is primarily an up-regulation of innate immunity precipitated by epithelial contact with a few ETEC-like bacteria. Rather it is likely a competitive exclusion event made possible by a large population of bacteria similar the ETEC pathogen, but unable to cause diarrheal disease.

Prior to conducting of this study, we conducted an experiment *in vitro* using the porcine jejunal cell line (IPEC-J2). We observed that in general, E. coli strains expressing LT in addition to the K88 adhesin bound to the IPEC-J2 cells in higher numbers than did *E. coli* expressing the adhesin alone (Koh et al., 2007; Mateo et al., 2007). Our *in vitro* study also indicated that $K88^+$ constructed ETEC strains could prevent the adherence of the pathogenic wild-type ETEC strain 3030-2 when the isogenic strains were administered in advance of the wild-type ETEC, and only when the strains expressed LT or a derivative of LT. Furthermore, this enhanced adherence phenomenon was observed not to be dependent upon toxigenic activity (i.e. biologically active A-subunit moiety; Mateo et al., 2007).

Several published studies indicate that LT contributes to bacterial colonization in the small intestine (Berberov et al., 2004; Allen et al., 2006; Zhang et al., 2006). Our observation that LT^+ laboratory constructs tended to adhere in higher numbers to the IPEC-J2 cells and was able to block adherence of wild-type ETEC is compatible with the observations of Berberov et al (2004) and Zhang et al. (2006) whose piglet challenge studies indicated that expression of LT significantly enhanced the colonizing ability of ETEC strains. Erume et al. (2008) conducted a study on gnotobiotic piglets to determine the contributions of LT and STb to the virulence of a K88+ ETEC strain. Their study confirmed the importance of LT as a virulence factor and indicated that LT was a greater contributor to virulence in K88 receptor-positive piglets than is STb (Erume et al., 2008).

In a subsequent weanling experiment conducted by our research group (Zhao et al., unpublished) in which pigs were inoculated twice (at 10 and 17 days of age) with the same isogenic constructs used in this study and challenged with ETEC strain 3030-2, a significant beneficial effect of the isogenic strains was demonstrated. Similar to the results of this study, control piglets developed severe ETEC-related diarrhea and dehydration, and most of the control pigs died or were euthanized before termination of the experiment. However, piglets inoculated with the isogenic constructs were protected and did not exhibit and ETEC-related clinical signs; in addition, piglets inoculated with the

isogenic constructs exhibited significant weight gain post-challenge, indicating that the constructs had a beneficial effect and may be a means of probiotic design (Zhao et al., unpublished).

Based of our preliminary investigations and current study, in developing a candidate ETEC probiotic/vaccine strain, it is important that the candidate strain expresses the necessary fimbrial adhesin (ie. K88ac) and a derivative of LT to enable the organism to efficiently adhere to the receptors in the small intestine yet not cause clinical signs of the disease. Timing of administration of the probiotic is also essential for its effectivity. Ideally, a probiotic should be administered before the anticipated period of exposure to the pathogenic bacteria in order for the probiotic to attach to the epithelial receptors and propagate. Results of this study indicate that $K88^+$ ETEC isogenic strains possessing attenuated LT and attenuated LT/STb have the ability to colonize the porcine small intestine and competitively exclude pathogenic ETEC at the level required to produce clinical disease. Finally, the use of avirulent ETEC strains may have significant advantages over the use of *Lactobacilli* strains which are commonly used in commercial probiotic cultures because these organisms would highly target the foci of ETEC infection.

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Tables and Figures

Table 1. *Escherichia coli* strains and plasmid used in this study.

Strain	Relevant properties	Plasmid vector	Source
1836-2	Field isolate, $K88^+$		Field isolate
8017	Negative control, $K88^+$	pBR322	Zhang *et al*, 2006
8221	LT (R192G), $K88^+$ LT mutant	pBR322	Zhang et al., unpublished
8488	LT (R192G), STb^+ $K88^+$ LT mutant	pBR322	Zhang et al., unpublished
C600N	Nalidixic acid resistant mutant of		unpublished
TK88-1	C600		unpublished
3030-2	C600N containing K88+ LT+		Field isolate
B44	plasmids		Smith and Halls, 1967
	Wild-type, $K88^+$ *ast*A^+ STb^+ LT^+ K99; F41; STa		

Table 3a. Mean total epithelial association index scores of bacteria from small intestinal Giemsa-stained segments from control pigs and pigs inoculated with strain 8488.

Pig ID	Treatment Group	Small Intestine Segment				Association Index[a]	
		Duodenum	Proximal Jejunum	Distal Jejunum	Ileum	Mean	Range
1-1A	Control	5	5	4	4	4-5	4.5
1-2A	Control	1	1	1	2	1-2	1.25
1-3A	Control	3	3	3	3	3	3
1-4A	Control	1	2	2	1	1-2	1.5
3-1	Control	4	3	4	4	3-4	3.75
3-2	Control	3	3	3	2	2-3	2.75
1-1	Control	2	2	4	3	2-4	2.75
1-7	Control	1	3	3	1	1-3	2
6-1	Control	3	2	2	2	2-3	2.25
6-4	Control	1	1	3	1	1-3	1.5
8-1	Control	3	2	3	3	2-3	2.75
7-1	Control	1	1	1	1	1	1
7-2	Control	3	3	3	2	2-3	2.75
2-1	8488	2	3	3	3	2-3	2.75
2-2	8488	1	1	2	1	1-2	1.25
2-3	8488	2	2	3	3	2-3	2.5
2-4	8488	1	3	2	3	1-3	2.25
3-3	8488	1	1	1	1	1	1
1-9	8488	2	1	2	1	1-2	1.5
1-10	8488	3	3	3	2	2-3	2.75
8-4	8488	1	2	2	1	1-2	1.5
7-9	8488	1	1	1	1	1	1
7-10	8488	1	1	1	1	1	1

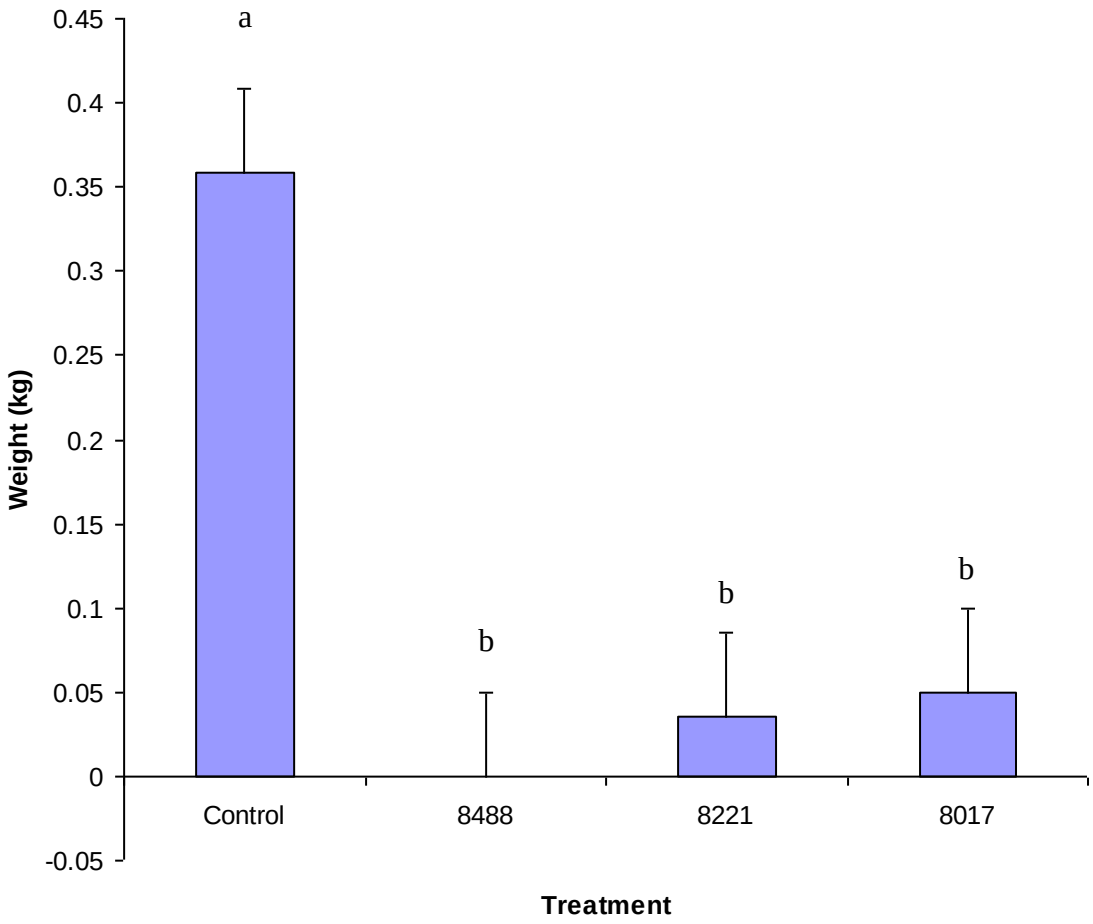
[a]The degree of bacterial adherence was determined on a scale of 1-5: 1, no villous-associated bacteria seen; 5, maximal bacterial along the entire length of the villi.

Table 3b. Mean total epithelial association index scores of bacteria from small intestinal Giemsa-stained segments from pigs inoculated with strains 8221 and 8488.

Pig ID	Treatment Group	Small Intestine Segment				Association Index[a]	
		Duodenum	Proximal Jejunum	Distal Jejunum	Ileum	Mean	Range
1-4	8221	1	1	1	1	1	1
1-5	8221	1	1	1	1	1	1
6-3	8221	1	1	1	1	1	1
6-6	8221	2	3	1	2	1-3	2
8-3	8221	1	1	2	2	1-2	1.5
7-6	8221	1	1	1	1	1	1
7-7	8221	1	1	1	1	1	1
7-8	8221	2	3	3	1	1-3	2.25
1-2	8017	1	1	1	1	1	1
1-3	8017	1	2	1	2	1-2	1.5
6-2	8017	1	1	3	1	1-3	1.5
6-5	8017	1	1	2	1	1-2	1.25
8-2	8017	4	3	3	3	3-4	3.25
7-3	8017	1	3	2	1	1-3	1.75
7-4	8017	1	3	2	2	1-3	2
7-5	8017	1	1	2	1	1-2	1.25

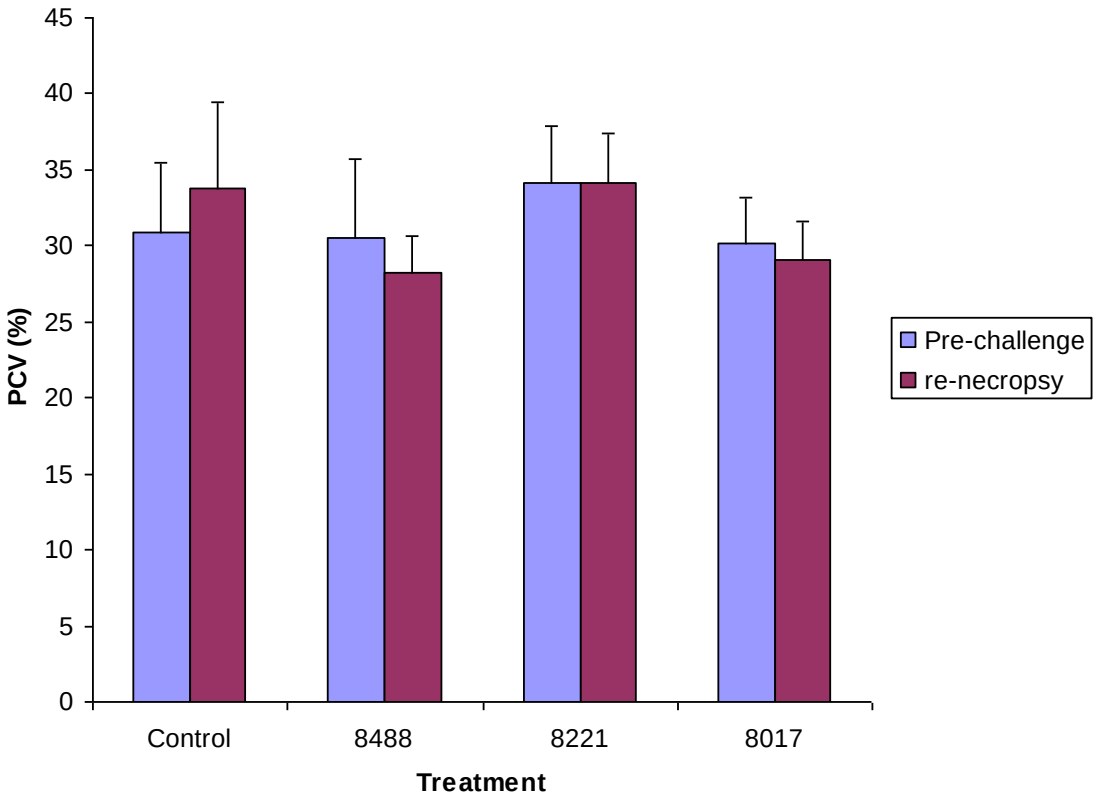
[a]The degree of bacterial adherence was determined on a scale of 1-5: 1, no villous-associated bacteria seen; 5, maximal bacterial along the entire length of the villi.

Fig 1. Comparison of total weight gain/loss of experimental piglets.



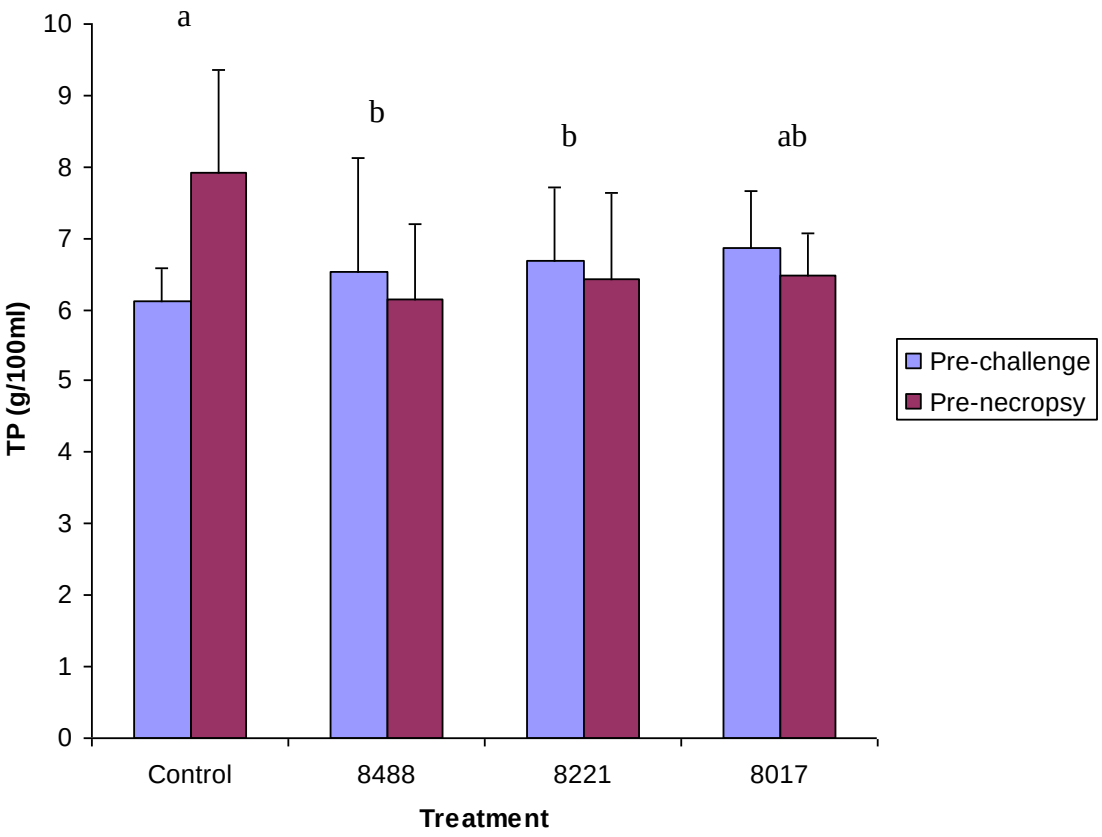
[a,b]Statistical analysis (Student's *t* test) indicated that weight loss of control piglets given only LB broth before challenge with ETEC wild-type strain 3030-2 was significantly higher than piglets pre-vaccinated with ETEC strains 8488, 8221 and 8017 ($P < 0.001$).

Fig 2. Pre- and post-challenge PCV values of experimental piglets.



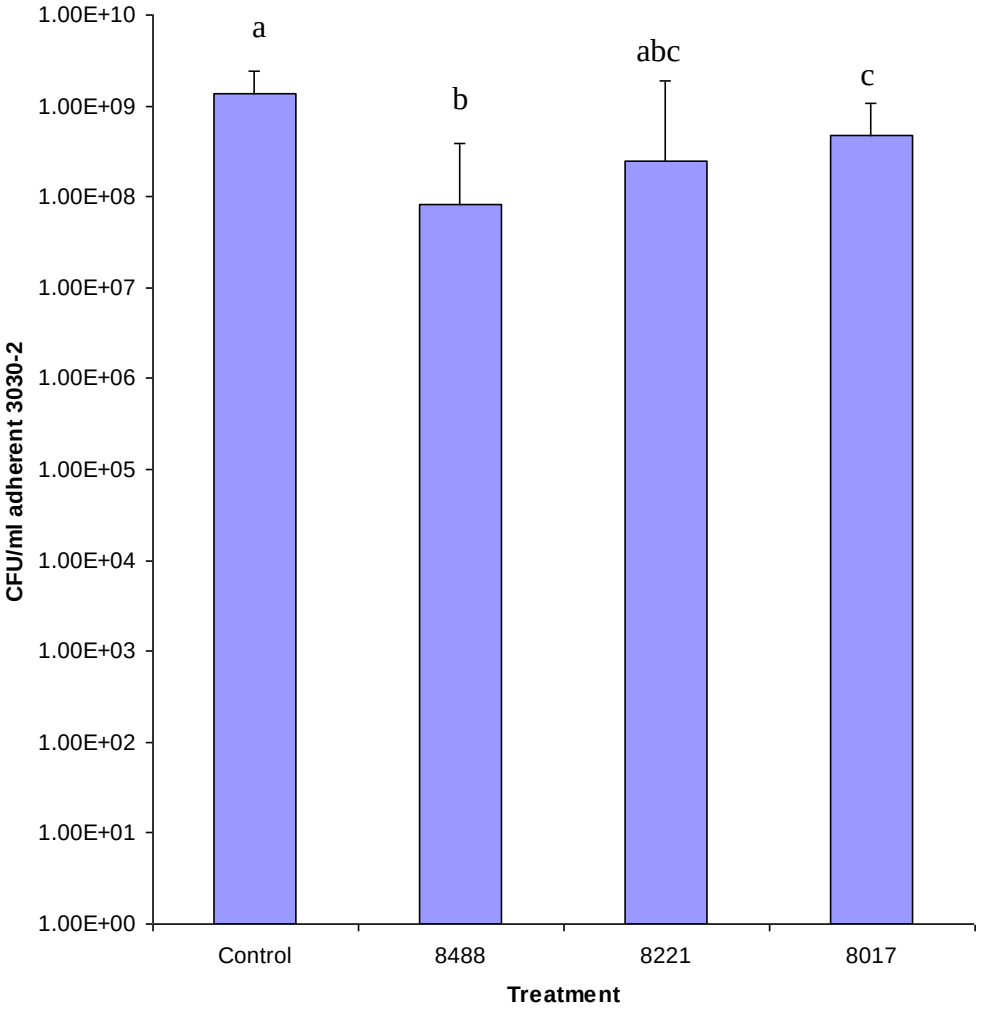
No statistical significance in PCV values was found between treatment groups.

Fig 3. Pre-and post-challenge plasma TP values of experimental piglets.



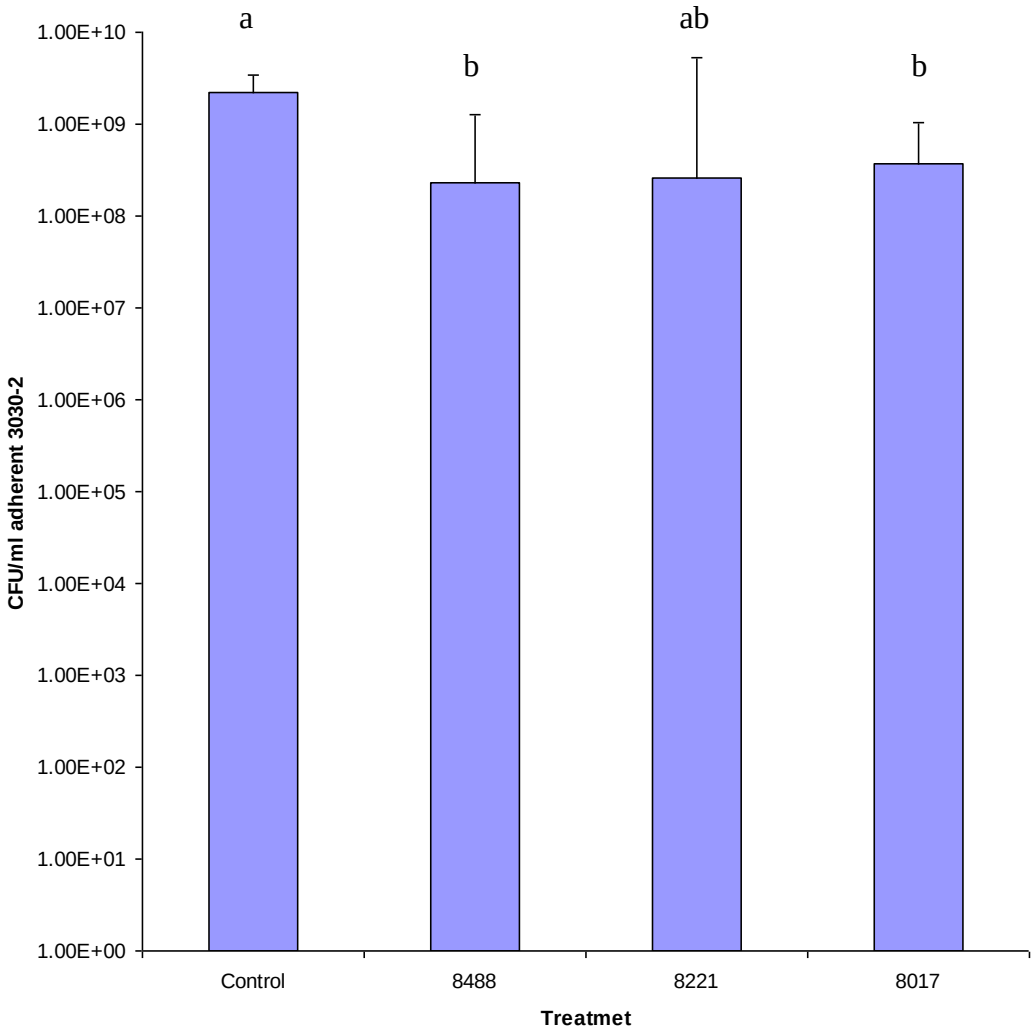
[a,b]Statistical analysis (Student's *t* test) indicated that the plasma TP of control piglets given only LB broth before challenge with ETEC wild-type strain 3030-2 was significantly higher than the plasma TP of piglets pre-vaccinated with ETEC strains 8488 and 8221 ($P < 0.001$).

Fig 4. Bacterial colonization measurements from the ileum of piglets inoculated with different isogenic ETEC strains and challenged with virulent ETEC strain 3030-2.



[abc]Statistical analysis (Student's *t* test) indicated that ileal colonization of strain 3030-2 in the control piglets inoculated with the CFU of control piglets was significantly greater that of piglets inoculated with strains 8488 and 8017 ($P = 0.002$ and 0.05, respectively); and ileal colonization of strain 3030-2 in piglets inoculated with strain 8017 was significantly greater that that of piglets inoculated with strain 8488 ($P = 0.05$).

Fig 5. Bacterial colonization measurements from the jejunum of piglets inoculated with different isogenic ETEC strains and challenged with virulent ETEC strain 3030-2.



[ab]Statistical analysis (Student's *t* test) indicated that jejunal colonization of strain 3030-2 in the control piglets inoculated with the CFU of control piglets was significantly greater that of piglets inoculated with strains 8488 and 8017 (*P* = 0.02 and 0.01, respectively).

Fig 6. Histological sections of the upper jejunum from A. control pig (ID # 1-1A), and B. pig inoculated with strain 8488 (ID # 2-1) and challenged with virulent strain 3030-2. Rabbit polyclonal antiserum against *E. coli* O157 somatic antigen was used as primary antibody and goat anti-rabbit IgG (heavy and light chain specific) was used for secondary antibody. Avidin-biotin alkaline phosphatase solution and Vector Red as the substrate were used to generate a red reaction product. Sections were stained immunohistochemically to demonstrate colonization of strain 3030-2 on the surface of the mucosal epithelium.

Figures removed due to e-mail size restrictions.