RESEARCH REPORT

SWINE HEALTH

Title: Effect of Probiotics on Enteric Colibacillosis – **NPB #98-052**

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I. Abstract:

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea and septicemia (toxic, blood-borne infection) in neonatal and recently weaned swine. A better understanding of the pathogenesis of enteric and septicemic colibacillosis is needed before more effective therapeutic and preventative measures can be developed. One aspect that is poorly understood is the mechanism by which bacteria spread from the intestine to the blood and other organs; this process has been termed translocation. Studies in laboratory animals have shown that translocation may be reduced by the presence of nonpathogenic indigenous bacteria, similar to the bacteria commonly found in probiotic preparations, in the intestine. Because the feeding of probiotics is a management strategy currently available to producers, we sought to determine whether the feeding of probiotic bacteria would reduce translocation of ETEC bacteria and the severity of enteric and septicemic colibacillosis in the gnotobiotic piglet model.

In the present study, *Lactobacillus acidophilus* and *Enterococcus faecium* strains were isolated from a commercial probiotic product for swine. *In vitro* competition experiments and *in vivo* challenge experiments were conducted with the probiotic strains and two serotype O8:K87:NM:F4ac ETEC challenge strains. The two ETEC strains included hemolytic strain WAM2317 and an isogenic nonhemolytic mutant, WAM2335. Neither probiotic bacterial strain was inhibitory to the growth of either ETEC strain in *in vitro* competition experiments. *In vivo* challenge experiments were conducted. Principal gnotobiotic piglets from 3 to 7 days of age were fed daily, 1-2 x 10^9 colony-forming-units (CFU) each of the *L. acidophilus* and *E. faecium* strains, whereas controls were fed sterile culture broth. Principals and controls were challenged at 9 days of age by feeding 1-2 x 10^9 CFU of WAM2317 or WAM2335. Severe weight loss and septicemia occurred only in phenotype A piglets, i.e., those susceptible to ETEC bacterial adherence due to binding of K88 (F4) fimbria. Microscopic examination of the small intestines of these pigs indicated that epithelial cells lining the villi had

sloughed, causing atrophy (shortening) of the villi. Epithelial sloughing was the result of ischemia (loss of blood supply to the region) caused by hypovolemia (decreased blood volume) and thrombosis; hypovolemia was the result of dehydration caused by diarrheal water loss. Severe dehydration in these piglets caused relative increases in serum concentrations of albumin, total protein, globulin, blood urea nitrogen, and creatinine. Severe metabolic acidosis with compensatorily increased concentrations of serum chloride and potassium were further manifestations of hypovolemic shock. The feeding of probiotic bacteria did not reduce the severity or progression of disease in phenotype A piglets, nor did it significantly reduce translocation of ETEC bacteria. Additional studies are needed to address the effects of probiotic bacteria inhibitory to ETEC on enteric colibacillosis, especially in piglets genetically predisposed to severe ETEC infection.

II. Introduction:

Diarrhea is the most important health problem in preweaned pigs and enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in this age group. Approximately 85% of ETEC diarrheal disease (enteric colibacillosis) cases in swine are caused by hemolytic strains that produce K88 (F4) fimbria. ETEC also commonly causes a condition known as septicemia in swine. Septicemia is an infection of the blood and various organs with resultant toxic manifestations. Results of one survey indicated that 14% of the cases of *E. coli* infection in swine involved septicemia and 16% of these isolates were ETEC strains. ETEC strains that cause septicemia or related conditions (e.g., acute death due to endotoxic shock) are most often hemolytic and express F4 fimbria. ETEC septicemia is secondary to intestinal ETEC infection and, hence, is also termed secondary septicemia.

The pathogenesis of ETEC-mediated secondary septicemia in swine is poorly understood. In general it is known that septicemia-inducing *E. coli* bacteria express virulence factors that mediate their spread from the intestine to other organs, and also their survival and growth in these locations. Two well-documented ETEC bacterial virulence factors that mediate diarrheal disease are fimbria and enterotoxin. Another potential virulence factor is hemolysin, but studies to date have not revealed its role, if any, in disease. In addition to bacterial virulence factors, host factors also heavily influence the outcome of infection. The presence of receptors for F4 fimbria on intestinal epithelial cells, an autosomal dominant trait, is a well-documented host factor that influences the severity of enteric colibacillosis in the pig. Another host factor that may influence the severity of disease is the presence of indigenous nonpathogenic bacteria in the intestine.

Studies in gnotobiotic mice have shown that the presence of indigenous nonpathogenic bacteria (e.g., *Lactobacillus* spp. and *Enterococcus* spp.) in the intestine reduces translocation, i.e., spread of bacteria from the intestine to extraintestinal sites, of other nonpathogenic bacteria, including non-pathogenic *E. coli* strains. However, studies addressing the effects of indigenous bacteria on translocation of pathogenic *E. coli* in mice have not been done, nor have studies addressing the effects of indigenous bacteria on translocation of either nonpathogenic or pathogenic *E. coli* in piglets. The

identification of bacterial flora that reduce translocation of ETEC bacteria in swine would be of practical importance, because they may potentially have the effect of preventing or reducing the development of secondary septicemia. In the present study, we hypothesized that the feeding of probiotic bacteria to neonatal gnotobiotic piglets would reduce the ability of ETEC bacteria to translocate, and would in effect reduce the development of *E. coli* septicemia. *E. coli* hemolysin is thought to play a role in protecting bacteria from phagocytic killing. Hence, we also hypothesized that ETEC strains producing hemolysin, in contrast to non-hemolytic strains, would have a greater capacity to spread from the intestine to the bloodstream, but this difference might only be evident in piglets with indigenous intestinal bacteria.

III. Objectives:

1. To determine the efficacy of probiotics for reducing the severity and progression of disease caused by enterotoxigenic *Escherichia coli* in swine.
2. To determine whether the feeding of probiotics to swine prevents the spread of enterotoxigenic *E. coli* from the intestine to the bloodstream and other organs.
3. To determine whether hemolysin enhances the ability of enterotoxigenic *E. coli* to establish a blood-borne infection in cesarean-derived, probiotic-fed pigs.

IV. Procedures:

Isolation of probiotic bacterial strains from a commercial product for swine. Approval to isolate and use for research probiotic bacterial strains from the commercial probiotic product Micro-Vet^{TM} SF Soluble was obtained from Boehringer Ingleheim (see letter dated 7/30/98, submitted earlier with Interim Report). Two probiotic bacterial strains, *Lactobacillus acidophilus* and *Enterococcus faecium*, were isolated from the product in MRS broth/agar (Difco) and KF Streptococcal broth/agar (Difco) cultures, respectively. Bacterial isolates were identified according to *Bergey's Manual of Determinative Bacteriology* using standard procedures, i.e., Gram stain, catalase test, ability to grow at 15 and 45°C, and the fermentation pattern displayed in the API system (BioMerieux).

***In vitro* competition experiments.** Three replicates of *in vitro* growth competition experiments between each of the two probiotic bacterial strains and each of two O8:K87:NM:F4ac ETEC strains (hemolytic strain WAM2317 and non-hemolytic mutant WAM2335; Moxley et al, 1998; Infect. Immun.66:5031) were conducted according to published procedures (Brashears et al, 1998; J. Food Prot. 61:66).

***In vivo* challenge experiments.** A total of 14 piglets from two litters were derived by cesarean section and maintained in isolator units by standard procedures. From 3 to 7 days of age, principal piglets were fed milk replacer containing 1 X 10^9 colony-forming units (CFU) of *L. acidophilus* and 2 X 10^9 CFU of *E. faecium* whereas, control piglets were fed milk replacer containing sterile trypticase soy broth (TSB; growth medium for the probiotic bacteria). At 8 days of age, the pigs were anesthetized by aseptic intramuscular injection of 20 mg of tiletamine hydrochloride-zolazepam hydrochloride (Telazol; Fort Dodge Labs, Fort Dodge, Iowa) per kg of body weight. While anesthetized, the pigs were weighed and checked for sterility or presence of the probiotic bacteria via culture of the nostrils and rectum. At this time also, blood samples were obtained aseptically from the heart by percutaneous needle aspirate. At 9 days of age, piglets were challenge inoculated by feeding 1 X 10^9 CFU WAM2317 or WAM2335 in the milk replacer. Every four hours following challenge, rectal temperatures were taken and piglets were examined for anorexia, diarrhea, depression, and clinical signs of septicemia, including a moribund condition. Every 24 hours after challenge, piglets were weighed. Piglets that became moribund were anesthetized, weighed, and blood sampled prior to euthanasia. Piglets that did not become moribund were monitored for 84-96 hours post-challenge before euthanasia and terminal sample collection. Piglets were necropsied immediately following euthanasia and tissues were collected for histopathology and bacterial culture. Tissues for histopathology were fixed in 10% neutral buffered formalin, processed by standard procedures, and microscopically examined. Sections of selected small intestinal tissue sections to detect the location of *E. coli* bacteria were processed for immunohistochemistry by standard procedures. Tissues for bacterial culture were collected using aseptic technique, and were ground, serially diluted, and plated on selective medium to quantitate *E. coli* bacteria. Brush border assays to test for F4 fimbrial adherence phenotype were conducted on small intestinal tissue specimens by standard procedures. Standard procedures were used to analyze serum samples for blood urea nitrogen (BUN), creatinine, sodium (Na^+),

potassium (K^+), chloride (Cl^-), bicarbonate (HCO_3^-), albumin, total protein, and globulin, and these tests were used to assess the severity and effects of dehydration.

V. Results:

Obj. 1. The probiotic bacterial strains did not reduce the severity or progression of disease caused by ETEC in swine.

Obj. 2. The probiotic bacterial strains did not prevent the spread of ETEC bacteria from the intestine to the bloodstream and other organs.

Obj. 3. Hemolysin did not enhance the ability of ETEC to establish a blood-borne infection in cesarean-derived, probiotic-fed piglets.

Detailed results of the experiments are provided below.

***In vitro* competition experiments.** The effects of probiotic bacteria on growth of the *E. coli* challenge strains *in vitro* are shown in Table. 1 (p. 6). Cultivation of either *L. acidophilus* or *E. faecium* with WAM2317 or WAM2335 did not reduce *E. coli* bacterial growth. Co-cultivation experiments involving both probiotic strains simultaneously with either ETEC challenge strain were not conducted. Probiotic bacterial growth controls (i.e., not co-cultivated with ETEC) included with each replicate displayed normal growth curves (data not shown).

***In vivo* challenge experiments.** All piglets developed diarrhea within 24 hours after ETEC challenge; however, only phenotype A piglets (i.e., those susceptible to F4 bacterial adherence to small intestinal epithelial cells based on the brush border assay) developed significant weight loss and systemic complications. Six of 9 phenotype A piglets, having a mean weight loss of 26.1%, became moribund from 31 to 93 hours post-challenge ($P < 0.005$). In contrast, none of 5 phenotype E piglets (i.e., those not susceptible to F4 fimbrial adherence) became moribund. Although phenotype E piglets developed mild diarrhea, they remained vigorous and gained weight (mean increase of 4.6%). Because piglets were euthanatized at different time points after challenge, the data was normalized to reflect the change in body weight per hour (Table 2, p. 7). The mean % change in body weight of phenotype A piglets per hour was 0.36, in contrast to 0.05 for the phenotype E piglets ($P < 0.01$). The number of *E. coli* in the liver ($\log_{10}$ CFU per g) and blood ($\log_{10}$ CFU per ml) was also significantly increased in phenotype A piglets (P 0.05). Six of 9 phenotype A piglets, in contrast to 0 of 5 phenotype E piglets, became bacteremic after challenge. Bacteremia occurred only in piglets that had developed severe (25-26%) weight loss and a moribund condition.

Histopathological examination of tissues and results of serum chemistries provided insight into the pathogenesis of secondary septicemia in the piglets. Microscopically in sections of small intestine from piglets with severe (25-26%) weight loss, epithelial cells on the distal tip, distal one-third, or complete length of many villi had sloughed. These lesions were indicative of ischemic bowel necrosis. Epithelial cell

sloughing exposed the underlying basement membranes directly to the intestinal lumen and also caused villus atrophy. Cores of lamina proprial stroma of affected villi were contracted and often contained foci of necrosis. Blood vessels in many organs including the intestines, liver, kidney, brain, and spleen contained fibrin-platelet thromboemboli, indicating that piglets with hypovolemic shock also had disseminated intravascular coagulation (DIC). Intestinal epithelial sloughing and DIC were seen only in piglets with severe weight loss and *E. coli* bacteremia; hence, ischemic bowel necrosis was interpreted to be the result of circulatory shock which in turn was due to the combined effects of hypovolemia and endotoxemia. Immunohistochemical staining of intestinal tissue sections with anti-O8 antiserum confirmed that the *E. coli* challenge strains in each phenotype A piglet had adhered to the intestinal epithelium and had adhered to exposed basement membranes in bacteremic piglets. In contrast, no O8-positive bacteria were found adherent to the small intestinal epithelium of phenotype E piglets. The three phenotype A piglets that did not develop bacteremia had few adherent O8-positive bacteria.

Similar to the clinical and histopathological findings, significant changes in the serum chemistries were associated with F4 adherence phenotype but not with probiotic feeding or challenge strain. Serum chemistries of piglets with severe weight loss and ischemic bowel necrosis supported the interpretation of hypovolemic shock. Statistically significant ($P < 0.005$) serum chemistry changes included increased concentrations of creatinine, albumin, total protein, and globulin, coupled with a decreased concentration of bicarbonate (HCO_3^-). Decreased concentrations of HCO_3^- in affected piglets caused an increase in the calculated anion gap and a mild relative increase in serum Cl^-. These changes reflected a state of metabolic acidosis. Serum chemistries were consistent with the expected effects of heat-labile enterotoxin (LT), produced both by WAM2317 and WAM2335. LT is known to induce loss of Na^+, Cl^-, and HCO_3^- in the feces. Increased concentrations of $Na^+$and Cl^- in the intestinal lumen further result in osmotic loss of water and diarrhea. Piglets with significant weight loss had severely decreased serum HCO_3^- concentrations, indicative of HCO_3^- loss in excess of that caused by lactic acidosis (i.e., in excess of that caused by poor tissue perfusion). Decreased serum HCO_3^- concentration (acidosis) caused mild relative increases in serum Cl^- (hyperchloremia) and K^+ (hyperkalemia). Water loss (dehydration) caused relative increases in serum albumin, total protein, and globulin, as well as pre-renal azotemia (moderately increased BUN and creatinine). Two piglets with markedly elevated BUN and creatinine also had histological evidence of renal tubular damage, indicating that the prolonged poor perfusion of the kidneys in these two piglets had begun to cause renal failure.

Table 1. Effect of probiotic bacteria on growth of *E. coli* challenge strains *in vitro*.[a]

Bacterial strains[b]	9 hr	12 hr	24 hr
WAM2335	8.81 ∀ 0.42	9.08 ∀ 0.08	9.17 ∀ 0.11
WAM2335 + *E. faecium*	8.78 ∀ 0.10	8.85 ∀ 0.22	8.98 ∀ 0.19
WAM2335 + *L. acidophilus*	9.12 ∀ 0.02	9.04 ∀ 0.00	8.93 ∀ 0.11
WAM2317	8.71 ∀ 0.04	9.01 ∀ 0.02	9.07 ∀ 0.04
WAM2317 + *E. faecium*	8.91 ∀ 0.08	8.99 ∀ 0.07	9.17 ∀ 0.12
WAM2317 + *L. acidophilus*	8.78 ∀ 0.16	8.98 ∀ 0.06	9.09 ∀ 0.09

[a]Number of *E. coli* ($\log_{10}$ CFU) per ml of growth medium. Results are the mean ∀ standard error of 3 to 6 replicated experiments.
[b]WAM2317 = nalidixic acid resistant mutant of porcine-origin hemolytic ETEC strain 2534-86. WAM2335 = isogenic nonhemolytic mutant of WAM2317. All cultures were grown to stationary phase in trypticase soy broth at 37EC. Hr = the number of hours of culture. In the case of *E. coli* plus probiotic strains, both organisms were present in the culture from 0 hours of incubation.

Table 2. Effect of probiotic bacteria on change in body weight and viable counts of enterotoxigenic *E. coli* in tissues of gnotobiotic pigs following challenge.

Treatment & Pig Phenotype (n)	❩%BW/hr[a]	Number of *E. coli* (log_{10} CFU/g)[b]				
WAM2317		Ileum	MLN[c]	Liver	Lung	Blood
Phenotype A (n =3)	-0.208_a	8.46_a	6.82_a	5.52_a	4.18_a	6.63_a
Probiotics + WAM2317						
Phenotype A (n =4)	-0.307_a	6.98_a	9.05_a	5.39_a	3.54_a	3.18_a
Phenotype E (n =2)	$+0.103_b$	7.5_a	7.33_a	3.16_a	3.81_a	0_b[d]
Probiotics + WAM2335						
Phenotype A (n =2)	-1.397_a	7.23_a	4.94_a	2.28_a	5.15_a	1.20_a
Phenotype E (n =3)	$+0.016_b$	8.09_a	6.82_a	2.12_a	2.94_a	0_b

[a]❩BW/hr = mean % change in body weight per hour after inoculation with *E. coli* challenge strain; means with different subscripts within a column are significantly different ($P < 0.05$).

[b]Geometric means of log_{10} colony forming units (CFU) of *E. coli* per gram of tissue or per ml of blood cultured on violet red bile agar. Means with different subscripts within a column are significantly different ($P < 0.05$).

[c]MLN = mesenteric lymph node.

[d]0 denotes no detectable growth.

Publications, Presentations, Abstracts

This work will be presented in the form of a poster at the Confererence of Research Workers in Animal Diseases, Nov. 7-9, 1999, in Chicago. The presentation is entitled, "Effect of Probiotic Bacteria on Translocation of Enterotoxigenic *Escherichia coli* in Gnotobiotic Piglets." The authors are E. Berberov, R. Moxley, R. Roscetti, M. Brashears, M. Scott, and D. Francis.

Summary

The feeding of probiotic bacteria isolated from a commercial product for swine did not reduce the severity of enteric colibacillosis nor the spread of enterotoxigenic *E. coli* (ETEC) bacteria from intestines to bloodstream in gnotobiotic piglets genetically susceptible to ETEC bacterial adherence. However, the probiotic strains used in this study also were not inhibitory to the growth of the ETEC challenge strains in *in vitro* competition experiments. The results indicate that the mere presence of nonpathogenic indigenous bacteria in the intestine by itself will not reduce translocation or ETEC nor the severity of disease caused by this organism in the neonatal piglet. ETEC bacterial translocation from intestine to peripheral blood was significantly associated with the development of circulatory shock, and shock developed only in piglets genetically susceptible to ETEC bacterial adherence in the small intestine. Additional studies are needed to address the effects of probiotic bacteria that are inhibitory to ETEC on enteric colibacillosis, especially in piglets genetically susceptible to ETEC infection.