RESEARCHREPORT
PORK SAFETY

Title: Effects of a commercial probiotic supplement on intestinal *E. coli* and growth in the weaned pig - **NPB #02-208**

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Abstract: Methods to optimize swine growth without the use of subtherapeutic antibiotics are currently desired. The use of a commercially available probiotic feed supplement was tested and compared to subtherapeutic antibiotic feeding for its effect on fecal *E. coli* concentrations, protection from *Salmonella* and rotavirus infections, and piglet growth under experimental and field conditions. Under experimental conditions, probiotic-fed piglets had higher total intestinal volatile fatty acid (VFA) concentrations than antibiotic-fed pigs, but similar to VFA concentration to control-fed pigs. Paradoxically, the total fecal *E. coli* concentration was significantly lower in the antibiotic-fed group compared to the two other groups. The prevalence of *Salmonella* and rotavirus following experimental challenge was similar in all groups. In the field study, fecal *E. coli* concentrations, prevalence of *Salmonella*, and growth production parameters among pigs fed probiotics, antibiotics, or control diets were all similar. Pigs fed antibiotics outperformed probiotic fed pigs, but the performance of the antibiotic fed pigs was not significantly different than the pigs on the control diet. Although the feeding of some probiotic bacteria to livestock are effective at achieving the desired outcomes, the results of this study suggest that the effectiveness of direct feed microbials is strain and possibly farm specific and highlights the need to individually validate probiotic formulations. Additional details concerning the mechanisms that probiotics and subtherapeutically fed antibiotics modulate the ecological balance of bacterial flora in the gastrointestinal tract are required in order to understand how the beneficial effects associated with certain feed additives are mediated.

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Introduction: Due to potential and perceived threats to food safety and public health, there is mounting public, political, and producer desire to identify alternatives to the use of subtherapeutic antibiotics in livestock production. One such option that has received increasing attention is the use of probiotics. Probiotics are defined as viable, nonpathogenic microorganisms that, when ingested, have beneficial effects in the prevention or treatment of several enteric disease conditions [1]. The mechanisms of action of probiotic bacteria remains unknown, but is believed that they act through the modification of the ecology of the intestinal microflora. Many probiotic fed-additives are commercially available for livestock. Regulations governing the licensure, sale, and addition of these products to animal feeds require only that the product be safe, without regard to product usefulness. The purpose of this study was to determine if the feeding of a specific probiotic-containing feed additive (Ultra Acidola Plus, Ultra BioLogics, Inc. Montreal Quebec, Canada) as recommended by the manufacturer would enhance protection against intestinal colonization of pathogens and/or enhance performance when compared to piglets fed a diet containing sub-therapeutic antibiotics or a control diet.

Objectives: We hypothesized that the addition of probiotic lactic acid bacteria would result in similar health and growth benefits as subtherapeutic use of antibiotics in the weaned pig. To test this hypothesis we compared the effects of dietary supplementation with probiotic lactic acid bacteria (LAB) with the use of subtherapeutic antibiotic (AB) use to enhance growth and protect against Salmonella and rotavirus infections in weaned pigs.

Procedures: The objectives of this study were carried out in two parallel experiments. The effects of LAB vs. AB on growth were measured in a longitudinal field trial conducted at an institutional swine facility (see Field Study below). The protection against *Salmonella* and rotavirus infection afforded by LAB and AB feeding was determined in controlled laboratory conditions (Challenge Study below). All studies were approved and conducted in accordance with Institutional Laboratory Animal Care and Use guidelines.

Field study

Research was conducted at the Ohio State University, Agricultural Technical Institute swine facility, Wooster OH, between September 2002 and October 2003. This facility has two nursery units, consisting of 8 slated floor raised pens in each room. Pens are arranged in two linear rows separated by a central walkway. Piglets born on location were managed in a conventional fashion (injected with iron supplement, vaccinated against erysipelas, atrophic rhinitis, Mycoplasma, and *Pastuerella multocida*, tails docked and, males castrated). Piglets were weaned at 21-24 days of age. During weeks when greater than 36 piglets were weaned, piglets were weight sorted into heavy and light classes based upon the mean weight of all pigs weaned that week. Five to eight pigs in each weight class were randomly assigned to receive the either the un-supplemented control ration, the control ration supplement with the probiotic (Ultra Acidola Plus), antibiotics. Probiotic-fed piglets first individually received an oral dose of 5 g probiotic at weaning followed by continuous in-feed dosing at 500g/ton during the first 3 weeks post-weaning while in nursery 1 (30ºC) and decreasing to 300g/ton feed when moved to nursery 2 (25ºC) for an additional 3 weeks. The complete mixed ration of the antibiotic-fed pigs contained lincomycin at 200 g/ton in nursery 1 and CSP (chlorotetracycline and sulfathiazole each at 50g/ton and penicillin at 25 g/ton). Feed and water were offered *ad libitum*. Feed consumption on a pen basis was recorded

daily. Pens were monitored daily for signs of illness or disease. Piglets that failed to thrive were removed form the study. Pigs were weighed at 3 and 6 weeks post weaning.

Weekly, fresh feces was collected from four to six areas on the floor of each study pen and pooled. Twenty-five grams of each pooled fecal sample homogenized with 225 ml of Buffered Peptone water (BPW) and analyzed for total coliforms, generic *E. coli* and generic Lactic Acid Bacteria (LAB) concentrations as described below. Samples were also cultured for *Salmonella* and assayed for the presence of rotavirus using a commercially available ELISA assay

Total *E. coli* concentrations were determined by plating serial dilutions of pooled fecal samples onto Violet Red Bile Agar containing 100mg/ml 4-methylumbelifeyrl-B-glucuronide[2]. Following overnight incubation at 37ºC, lactose-positive (total coliforms) and lactose-positive:MUG-positive (presumptive *E. coli*) colonies were enumerated aided by UV illumination. Total *Lactobacilli* concentrations will be determined by spread plating serial dilutions of fecal samples on ROGOSA agar[3]. ROGOSA plates were incubated anaerobically at 37ºC for 48 h prior to enumeration of colonies. *Salmonella* detection was performed by pre-enriching the BPW fecal homogenates overnight at 37º and subsequently transferring 1 ml of pre-enrichment to 9 ml of Tetrathionate broth (TT). TT broth was incubated overnight at 37ºC and 0.1 ml subsequently transferred into 10 ml of Rappaport-Vassiliadis (RV) broth. After overnight enrichment at 37ºC, RV was struck to XLT-4 media for isolation . Black colonies appearing on XLT-4 following 48 hr of incubation at 37ºC were considered salmonella suspect and confirmed using typical reactions in TSI and urea agar and agglutination with serogroup specific antisera.

Challenge study

On five separate occasions, groups (21-30 animals) of weaned pigs were transported to isolation facilities at the Food Animal Health Research Program. Piglets of each weight class were randomly assigned to one of three treatment groups as described above. Groups of animals (7-10 pigs) were housed in solid floor pens that were cleaned daily. Feed (according to the treatment group assignment) and water was provided *ad libitum*. Following a one-week acclimation period, groups of piglets were challenged with either *Salmonella* (Challenge experiments 1 and 2) or rotavirus (challenge experiment 3 to 5). Pigs were monitored daily for clinical signs of disease.

For the *Salmonella* challenges, five ml of an overnight broth culture of a porcine-origin, multidrug-resistant *Salmonella enterica* Thyphimurium was administered orally to one piglet in each fed group. The challenged piglet was co-housed and allowed to immediately co-mingle with the other piglets in the group. Feces, collect per rectum on cotton-tipped applicators, from each animal in the challenge group on days –3, 0, 1,3, 7 and 10 post challenge were analyzed quantitatively for coliforms, *E. coli*, and LAB (as described above) and for *Salmonella*. Serial dilutions of the BPW homongenates were plated on 150 mm XLT-4 plates containing antibiotics and incubated overnight at 37ºC. Black colonies were enumerated and 10% of colonies confirmed as *Salmonella* based on TSI and urea reactions. In addition, to detect *Salmonella* below the detection threshold of the direct plating method, enrichment cultures, as described above, was also performed.

VFA content of ingesta from the proximal colons of challenge study pigs was determined using the method previously described by VanWinsen et al.[4]

For the rotavirus challenges, three identical experiments were performed. Piglet assignment to treatment group and animal husbandry was identical to that described for the *Salmonella* challenges. Piglets were challenged with a porcine rotavirus by oral innocualtion of each piglet. Feces, collected per rectum on cotton-tipped applicators,

from each animal in the challenge group on days –3, 0, 1,3, 7 and 10 were analyzed quantitatively for coliforms, *E. coli*, and LAB (as described above) and for rotaviruses. On days 3, 7 and 10, two piglets from each group were sacrificed and necropzied to detect lesions compatible with rotavirus infection and obtain intestinal contents for VFA and viral analysis.

Statistical Analyses

Multiple comparisons between treatment groups were made using repeated measures ANOVA procedure in SAS for rank-transformed microbiological parameters (SAS Institute, Carey, NC). Generalized linear models (GLM) followed by Tukey's test for multiple comparisons for the production data and VFA data from challenge studies. Bi-variate analyses of correlation between coliform, *E. coli*, and LAB concentrations, VFAs, and production parameters were performed using the Pearson Product-Moment correlation coefficient. Statistical significance for type I error was set at 0.05.

Results:

Field Study

A total of 51 pens of animals were enrolled in this study, representing 17 pens per treatment-group. Eight replicates of the each treatment group comparisons involved "heavy" class animals and an equal number of pens contained "light" class animals. On one occasion there were insufficient piglets to allow for weight sorting, so all available pigs weaned that week were randomly assigned to one of the three treatment groups, without weight sorting.

Production data results are outlined in Table 1. Although animals were randomly assigned to treatment groups, the probiotic incoming weight of pigs in the probiotic treatment group was slightly, but significantly, lower than the incoming weight of pigs in the other two groups. There was no difference in fecal coliforms, *E. coli,* LAB or VFA in treatment pens ($p>0.05$).

As expected, *E. coli* and coliform concentrations were strongly correlated. Likewise, expected correlations were observed between feed consumption, weight gain and ADG. LAB concentrations were not significantly correlated with either total coliforms ($r=0.03$, $p=0.79$) or *E. coli* ($r=0.19$, $p=0.16$). Coliforms and *E. coli* were however (negatively) correlated with feed consumption ($r=-0.45$, $p=0.001$ and $r=-0.36$, $p=0.009$), and consequently also with feed conversion ($r=-0.42$, $p=0.002$ and $r=-0.49$, $p=0.0003$).

Salmonella sp. was detected in 16/333 (4.8%) pens sampled. There were no significant differences in *Salmonella* prevalence between treatment groups in the field study. Four of 184 (2%) of samples tested positive for rotaviruses-three from probiotic fed animals and one from an antibiotic-fed animal. The small number of rotavirus-positive samples limited interpretation of the effects of treatment on rotavirus excretion.

Laboratory Challenge: Five separate pathogen challenge experiments were conducted, the first two involving only *Salmonella* challenge and the remainder involving rotavirus. Oral inoculation of a single piglet in each group with Salmonella typhimurium resulted in dissemination of the pathogen to every other pig in the pen. Daily prevalence of salmonella in each pen varied daily and ranged between 12.5-100%. Average *Salmonella* prevalence values over the two experiments are depicted in Figure 1. The *overall* number of *Salmonella*-positive samples was highest from the probiotic fed groups of animals: 46/54, 31/49 and 35/54 for probiotic-fed, control-fed and antibiotic-fed animals respectively. However, this small disparity did NOT result in statistically significant differences in prevalence values when the large within-pen and

between-pen variation components were included in the repeated-measures statistical analyses.

Total volatile fatty acid concentrations was significantly lower in ingesta collected from antibiotic fed pigs than probiotic fed pigs (Table 2). However, the partitioning of VFA types was similar in all groups. Coliform and *E. coli* concentrations were significantly correlated. LAB concentrations were correlated with total coliforms, but no significant correlations between any bacterial parameter measured and the total VFA concentration was observed.

Interpretation and Application of Results

There are many products on the market sold as probiotics with label claims to enhance growth and production. Regulations governing the licensure, sale, and addition of these products to animal feeds require only that the product be safe, without regard to product usefulness. How probiotics act to modulate the ecology of the gastrointestinal tract is poorly understood. The addition of some probiotics, especially lactic acid-producing bacteria such as the ones included in this probiotic mixture have been shown to provide benefical effects such as enhanced weigh gain and feed conversion and protection from infection in pigs and other animals [5-8]. One the other hand, other researchers have tried to identify beneficial effects of other probiotic bacteria without success[9-12]. In fact, in one study in dogs, animals actually excreted more *Salmonella* and *Campylobacter* following treatment with probiotics[13]. Importantly, most studies that fail to find beneficial effects of probiotic feed rarely end up in the scientifically peer-reviewed literature, thus the balance of readily available literature tends to support widespread effectiveness of probiotic feeding. Presently, since we do not know how these products work, it is a hit-or-miss approach to achieving favourable food safety, animal health and production effects by incorporating them in the ration.

It is been previously hypothesized that the effects of LAB are modulated primarily through the production of short-chain or volatile fatty acids that have a detrimental effect on Gram-negative bacterial flora (primarily coliforms and *E. coli*). Data from this study and other recently published literature however suggest that this mechanistic view is oversimplified. LAB are known to produce VFAs[14]. Although probiotic fed pigs in the experimental challenge had higher total VFA concentrations, no correlation were found between VFAs and *E. coli* or coliforms concentrations in this study . This is in accord with another recently published study conducted in piglets with a different probiotic preparation [15]. Coliform and *E. coli* concentrations generally increased as the animals aged. This may account for the differences in counts observed in the experimental and field studies. The experimental studies, the pigs were followed only for 3 weeks post weaning whereas in the field study pigs were tested until they left the nursery. It is of interest to note that the *E. coli* and coliform concentrations were significantly (negatively) correlated with feed consumption (*i.e.* pigs that ate more had less *E. coli).* From this study it is not possible to determine if high concentrations of these organisms are a *direct* result of less feed consumption and an unhealthy balance of microorganisms in the intestine or whether high *E. coli* concentrations actually contribute to the cause of decreased appetite and feed conversion.

Since the gastrointestinal microbiology of piglets may vary significantly depending upon environment, health, and other management factors it is presently not possible to guarantee or predict the effectiveness of a particular probiotic treatment on individual farm even it has been previously assayed under different management conditions. In order to consistently select and predict the potential beneficial effects of a particular probiotic treatment, the mechanism of action must first be established.

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Table 1. Growth, production and microbiological parameters of weaned pigs measured in the field study. Numbers in the same row with different superscripts significantly different (P<0.05).

Parameter	Control		Antibiotic		Probiotic	
Initial weight (lbs)	14.65	a	14.11	a,b	**13.86**	b
Weight at 6 weeks (lbs)	26.30	a	25.29	a	25.03	a
Weight at 9 weeks (lbs)	52.79	a	**55.63**	b	51.77	a
Feed consumed in Hot Nursery (lbs)	16.63	a	17.52	a	16.21	a
Feed consumed in Cold Nursery (lbs)	58.14	a,b	**61.64**	a	56.19	b
Feed consumed total (lbs)	74.78	a,b	**79.16**	a	72.40	b
Weight gain in Hot Nursery (lbs)	11.19	a	11.65	a	11.17	a
Weight gain in Cold Nursery(lbs)	27.49	a	**29.32**	b	26.73	a
Total overall weight gain (lbs)	38.68	a,b	**40.98**	a	37.91	b
Average Daily Gain in Hot Nursery	0.53	a	0.55	a	0.53	a
Average Daily Gain in Cold Nursery	1.31	a	**1.39**	b	1.27	a
Average Daily Gain -Overall	0.92	a,b	**0.97**	a	0.90	b
Feed Conversion in Hot Nursery	1.48	a	1.51	a	1.45	a
Feed Conversion in Cold Nursery	2.12	a	2.11	a	2.11	a
Feed Conversion –Overall	1.93	a	1.93	a	1.91	a
Fecal Coliforms (Log_{10} CFU/g)	6.49	a	6.31	a	6.39	a
Fecal *E. coli* (Log_{10} CFU/g)	6.05	a	5.64	a	6.08	a
Fecal LAB (Log_{10} CFU/g)	10.57	a	10.55	a	10.59	a

Table 2. Microbiological and VFA parameters of weaned pigs measured in the experimental challenge. Numbers in the same row with different superscripts significantly different (P<0.05).

Parameter	Control		Antibiotic		Probiotic	
Fecal Coliforms (Log_{10} CFU /g)	6.80	a	5.33	a	6.73	a
Fecal *E. coli* (Log_{10} CFU /g)	4.08	a	3.32	a	3.86	a
Fecal LAB (Log_{10} CFU /g)	8.93	a	8.74	a	8.89	a
Total Volatile Fatty Acids (µmole/ml)	113.9	a,b	**104.6**	a	**128.6**	b
% Acetic	60.3	a	61.6	a	60.6	a
% Proprionic	25.8	a	26.0	a	24.7	a
% Butyric	11.7	a	10.6	a	12.6	a
% Isobutyric	0.3	a	0.3	a	0.2	a
% Valeric	1.6	a	1.2	a	1.7	a
% Isovaleric	0.3	a	0.3	a	0.2	a

Figure 1. Prevalence of *Salmonella* in pigs feed subtherapeutic antibiotics, probiotic lactic acid bacteria or control diets.