RESEARCH REPORT
PORK SAFETY

Title: Evaluation of antimicrobial alternatives to reduce the development of antibiotic resistance - NPB#02-084

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Abstract: A 35-day growth assay was conducted to assess the effect of inorganic minerals and probiotic feed additives on growth performance and development of antibiotic resistance in nursery pigs. Ninety six pigs, with an average weight of 13.11 pounds and ranging in age from 18 to 20 days of age were randomly assigned to six experimental treatments. Experimental treatments consisted of a non-medicated basal diet (control diet), and diets supplemented with Mecadox at 50 g/ton (positive control), copper sulfate at 250 ppm and zinc oxide at 3000 ppm. Two diets were supplemented with BioPlus 2B®, a probiotic feed additive consisting of spores of *Bacillus* lic*heniformis and Bacillus subtilis* at 1.1 million spores/gram of feed and 1.3 million spores/gram of feed, respectively. Pigs were weighed weekly to assess growth. Feed added to feeders was recorded and feeder weights were obtained on days 21 and 35 of the study to assess feed consumption. Rectal swabs were collected from pigs on days 0, 21 and 35 of the study for isolation of enteric bacteria belonging to the genus *Enterococcus* to determine antibiotic resistance resulting from the experimental treatments. The non-antimicrobial feed additives failed to improve growth rate or feed efficiency in comparison to the non-medicated control diet. Furthermore, the diet supplemented with Mecadox failed to support an increase in growth rate compared to the control diet. The non-antimicrobial feed additives stimulated an increase in feed consumption of the nursery diets with the greatest feed intakes associated with the diet supplemented with zinc oxide ($P<0.05$). Consumption of the non-antimicrobial feed additives did not result in an elevation of minimum inhibitory concentrations (MIC) above base line levels measured at the initiation of the study. Numerical increases in MIC were observed for the control and Mecadox-supplemented diets, however, the increases failed to achieve significance ($P>0.05$). With regards to performance, results of the present study concur with results of other studies where growth responses to non-antimicrobial feed additives tend to be variable, with improvements in feed intake and feed efficiency being observed more commonly. With regards to antibiotic resistance, consumption of the non-antimicrobial feed additives did not promote an increase in resistance of enterococcus isolates to vancomycin.

Introduction: The ability of bacteria to resist the action of antibiotics has evolved to become a worldwide public health issue. Human health professionals have implicated the practice of supplementing livestock diets with growth promoting antibiotics as a major factor in the current antibiotic resistance problem. This opinion has fostered the suggestion that feeding antibiotics to livestock at growth promoting levels should be banned. To date, legislation proposing removal of growth promoting antibiotics from the production environment has been put forth, but has failed to be passed into law. Antibiotic resistance arises as a result of an innate tendency of bacteria to acquire and retain genes that indefinitely confer the ability to resist the action of antibiotics. Langlois et al. (1988) demonstrated that complete withdrawal of antibiotics from the production environment results in a decrease in bacterial resistance of fecal coliforms. The length of antibiotic withdrawal from the population under study was approximately 10.5 years. In spite of the extended withdrawal period, the level of bacterial resistance to tetracycline and level of multiple antibiotic resistance documented was significant and indicated that a stable, resistant microflora persisted from previous antibiotic exposure. The observation that antibiotic withdrawal promotes a reduction in the ability of bacteria to resist antibiotics is highly significant. This fact has further been demonstrated in Denmark where a ban on growth promoting antibiotics has resulted in decreased prevalence of resistant bacteria in the swine production environment (Aaerstrup et al., 2001). Antibiotics continue to have a legitimate place in the production of healthy hogs, therefore, effectively addressing the problem of antimicrobial resistance requires more judicious use of antibiotics in animal production. Use of antibiotic alternatives in diet formulation and in accordance with appropriate management, sanitation and biosecurity measures may provide a vehicle for reducing the use of feed-grade antibiotics in swine production.

Alternatives to the use of antibiotics in animal production continue to evolve and some that have shown promise for reducing antibiotic usage in swine production include inorganic mineral supplementation and probiotic supplementation of nursery diets. Zinc, in the form of zinc oxide, and copper, in the form of copper sulfate are used in the post-weaning phase of swine production to promote growth and prevent the occurrence of certain enteric diseases. When used to promote growth and/or prevent disease, both minerals are added to the diet at pharmacological levels, which are in excess of nutrient requirements of the weaned pig. Zinc oxide supplementation of nursery diets to levels ranging from 1500 to 3000 ppm zinc (Hill et al., 2000; Hill et al., 2001) and copper supplementation to 250 ppm copper (Cromwell et al., 1998; Cromwell, 2001) have been shown to support improved nursery pig performance. Probiotics, also known as direct fed microbials have received considerable attention with regards to their use as an alternative to antibiotics in livestock feeding. A compilation of studies with pigs demonstrate an overall benefit to feeding probiotics, but inconsistencies have also been reported (Cromwell, 2001). Studies in Europe with a probiotic consisting of *Bacillus* spores demonstrated favorable results on performance and pathogen control. Zani et al. (1998) and Kyriakis et al. (1999) both showed improvements in growth and feed efficiency, in addition to decreased incidence of post-weaning diarrhea in nursery pigs. In both studies, the decreased incidence of diarrhea was associated with a decrease in recovery of *E. coli* from pigs fed probiotics. Letellier, et al. (2000) showed that *Salmonella* carriage in orally challenged pigs could be effectively reduced with probiotics, and probiotics plus a fructooligosaccharide prebiotic reduced carriage and shedding of *Salmonella*. The propensity of probiotics to reduce carriage and excretion of enteric pathogens may also have post-harvest food safety implications.

In summary, antibiotic supplementation of livestock diets to promote improved performance is being challenged on the grounds that the practice negatively influences public health. Removing growth promoting antibiotics from the swine production environment has been shown to decrease the prevalence of resistant bacteria associated with pigs. However, this has resulted in the need for increased therapeutic use of antibiotics. Removing growth promoting antibiotics from the commercial production environment will require feed additives that can effectively replace antibiotics in diet formulation. Studies with inorganic minerals and probiotics suggest that these compounds may have applications in growth promotion and minimizing certain diseases of pigs, in addition to facilitating reductions in the use of antibiotics in swine production.

Objectives: The overall objective of the study was identification of viable alternatives to antibiotic feeding in swine production that will continue to promote the sustainability of the U.S. swine industry, and control the progressive development of bacterial resistance to antibiotics. The central hypothesis of the study was the effective control of the progression of bacterial resistance is predicated on reducing the routine usage of growth promoting antibiotics in swine feeding programs without sacrificing animal health. Two research objectives were developed. The first research objective was assessment of animal health and performance in response to inorganic mineral supplementation and probiotic supplementation of swine diets. The working hypothesis for this objective was acceptance of alternative diet formulation strategies require documentation that proper application can sustain acceptable production and limit the influence of disease. The second research objective was assessment of inorganic mineral supplementation and probiotic supplementation of swine diets on the development of bacterial resistance. The working hypothesis for this objective was inorganic minerals and probiotics utilize mechanisms of action to promote health and growth performance that differ from antibiotics. Therefore, these compounds should not contribute to the development of bacterial resistance in response to feeding and provide a tool to effect reductions in antibiotic usage and resistance.

Procedures:

Objective 1. A 35-d growth assay was used to assess the effect of inorganic mineral supplementation and probiotic supplementation of swine diets on pig performance. The experimental design consisted of a randomized complete block design and was replicated three times for a total of four blocks. The pen constituted the experimental unit. Six dietary treatments were used in the study. Negative and positive control diets, respectively, consisted of a non-medicated, corn-soybean meal basal diet (negative control) and Carbadox was added to the basal diet at 50 g/ton (positive control diet). Copper and zinc, respectively, were added to the basal diet in their inorganic forms at pharmacological levels. The basal diet was supplemented with copper sulfate to 250 ppm copper. The basal diet was supplemented with zinc oxide to 3000 ppm zinc. *Bacillus* spores (*B. licheniformis, B. subtilis*) of the probiotic BioPlus 2B® were added to the basal diet at two levels, based on recommended levels of dietary inclusion in the United States and Europe. The basal diet was supplemented to 1.1 million *Bacillus* spores/gram of feed (United States standard) and 1.3 million *Bacillus* spores/gram of feed (European standard). A phase feeding protocol was used and the first nursery diet (N1) was fed for the initial 21 days and the second nursery diet (N2) fed for the remaining 14 days. Ninety six crossbred barrows and gilts, with an average bodyweight of 13.11 pounds and weaned at 18-20 days of age were assigned to the six experimental diets. Enrollment of pigs in the study was based on sows and the litters of

prospective sows not having been treated with antibiotics during the lactation period. Pigs were housed in floor pens in an environmentally controlled nursery. The pens were 22 square feet in size, housed four pigs per pen and each pen was fitted with plywood partitions to prevent fecal contamination from adjacent pens. Bodyweight measurements were obtained weekly. A feed record was maintained and feeder weights were obtained on day 21, which corresponded to the change from diet N1 to diet N2. Feeder weights were also obtained on day 35 of the study, which corresponded to termination of the growth and feed performance aspect of the study. Least square means for growth and feed performance data were calculated and subjected to the GLM procedure of SAS®. The least significant difference test was used as the mean separation procedure.

Objective 2. Enterococcus was cultured from pigs and used as the sentinel organism to evaluate the development of antibiotic resistance. *Enterococcus* was selected as the sentinel organism due to its frequent association with outbreaks of antibiotic resistant bacteria in human intensive care facilities (Huovinen, 1999). Rectal swabs were collected from pigs on days 0, 21, and 35 from pigs enrolled in the study. Swabs were transported to the microbiological laboratory, homogenized in 0.3 mls of sterile water and a sample streaked onto bile esculin agar plates to obtain individual colonies. Bile esculin agar is a selective media for cultivation of *Enterococcus* species. Bile esculin agar plates were incubated aerobically at 35^0C for a period of 24 hours. Isolated colonies demonstrating hydrolysis of esculin were transferred to blood agar plates and incubated aerobically at 35^0C to obtain pure colonies. After obtaining pure colonies, identification of *Enterococcus* to the species level was accomplished using the AP120E biochemical analysis system, a system of enzymatic and fermentation reactions used for identification of bacteria. The broth microdilution assay was used to determine susceptibility of enterococcus isolates to vancomycin, the antibiotic of choice for treating enterococcus infections in humans. Concentrations of vancomycin used in the microdilution assay to screen the enterococcus isolates for resistance were 2, 4, 8, 16, 32, 64, 128 and 256 µg/ml. The vancomycin concentrations selected were identical to concentrations used in the microbiological laboratory of the local human medical center. 96-well plates were prepared, inoculated and incubated at 35^0C and evaluated after a full 24 hours of incubation. Minimum inhibitory concentrations equal to or greater than 32 µg/dl were considered resistant. Least square means for MIC were calculated and subjected to the GLM procedure of SAS®. The least significant difference test was used as the mean separation procedure.

Results:

Objective 1. Growth performance data is shown in Table 1 and feed consumption and feed efficiency data is shown in Table 2. The feed additives under evaluation failed to improve weight gain measured at day 21 and day 35 of the study when compared to the non-medicated control. A similar trend was apparent for average daily gain calculated for the duration of study and for the periods encompassing day 0-21 and day 21-35. The lack of a growth response to the diet supplemented with Mecadox was unexpected and raises questions about health of the pigs, since high health status pigs tend to demonstrate poor responses to growth promoting antibiotics. Feed additives failed to improve feed consumption per pound of bodyweight gain (feed/gain) when compared to the non-medicated control. A response to feed additive supplementation was observed when feed intake was evaluated. Pigs fed the diets supplemented with zinc oxide tended to consume more feed compared to the non-medicated control ($P<0.05$). Consumption of the Mecadox supplemented diet was similar ($P>0.05$) to the diets

containing the probiotics and consumption of the diet containing copper sulfate was similar (P>0.05) to the control diet.

Relevance to the producer. All of the feed additives evaluated failed to improve growth rate or feed efficiency compared to the non-medicated control diet. The improvement in feed intake was most consistent for the diets supplemented with zinc oxide, which is used extensively in nursery diet formulation in conjunction with growth promoting antibiotics. The results of the present study agree with other studies where growth responses to many feed additives suggested as alternatives to feed-grade antibiotics are inconsistent depending on the product. The effect of improvement in feed consumption is also consistent with previous studies where the beneficial effect of non-antimicrobial feed additives tends to be associated with improvements in feed consumption and feed efficiency. Based on the conditions of the study, the results of the growth assay suggest that the ability of non-antimicrobial feed additives to replace feed-grade antibiotics in swine production is debatable and requires further evaluation.

Objective 2. Mean resistance of enterococcal isolates collected prior to consumption of experimental diets (Day 0) and after consumption of experimental diets (Days 21 and 35) are shown in Table 3. Isolates collected on Days 21 and 35 of the study were combined for analysis. Enterococcus isolates were identified to the species level, and in rank order consisted of *E. faecium, E. faecalis, E. avium, E. durans* and *E. gallinarum*, respectively. Mean resistance on Day 0, prior to the pigs consuming the experimental diets, was similar (P>0.05) across all dietary treatments, except for the BioPlus 2B® diet with 1.3 million spores/gram of feed (BP 2). The MIC for this treatment was significantly (P<0.05) higher than the other experimental treatments, but the MIC still resided in the susceptible range. Mean resistance on Days 21 and 35 revealed that the MIC of the six experimental diets was similar (P>0.05). However, there were numerical increases in the MIC of the non-medicated, negative control diet and the medicated, positive control diet (Mecadox) that failed to achieve significance. The decrease in MIC of the BP 2 diet was remarkable. The use of probiotic compounds, especially those containing enterococcus isolates in humans has raised concerns that these products actually contribute to the risk of transfer of antibiotic resistance elements to humans. However, the BioPlus 2B® product is different from many probiotic compounds because it consists of spores of *Bacillus subtilis* and *Bacillus licheniformis*. The explanation for the beneficial effect of the BP 2 diet on MIC is unclear, but speculatively, the probiotic may have limited colonization of the resistant isolates by effectively altering the intestinal ecosystem, making it difficult for the bacteria to survive.

Relevance to the producer. The MIC data indicate that the non-antimicrobial feed additives evaluated in the study did not increase the resistance of the enterococcus isolates to vancomycin. The MIC data also appear to indicate that pigs do not naturally harbor resistant populations of enterococci in their digestive tract. Therefore, more research is necessary to clarify the impact of animal production practices on antibiotic resistance and public health. Based on the conditions of the study, the MIC data suggest that curbing the development of resistant bacteria in the production environment through the use of non-antimicrobial feed additives appears to be possible.

Table 1 – Weight gain and average daily gain

Item	BP 1	BP 2	Control	$CuSO_4$	Mecadox	ZnO	SD
Day 0 weight, lb	13.09	13.12	13.08	13.12	13.12	13.13	0.33
Day 21 weight, lb	22.51	22.16	21.67	22.27	23.63	24.71	3.17
Day 35 weight, lb	36.90	37.11	36.03	36.06	39.08	40.29	5.13
ADG Day 0-35, lb/d	0.68	0.68	0.65	0.65	0.74	0.78	0.14
ADG Day 0-21, lb/d	0.45	0.43	0.41	0.43	0.50	0.55	0.15
ADG Day 21-35, lb/d	1.03	1.07	1.03	0.98	1.10	1.11	0.22

BP 1 = BioPlus 2B at 1.1 million spores/gram of feed
BP 2 = BioPlus 2B at 1.3 million spores/gram of feed
$CuSO_4$ = Cooper sulfate
ZnO = Zinc oxide
ADG = Average daily gain

Table 2 – Feed consumption and feed efficiency

Item	BP 1	BP 2	Control	$CuSO_4$	Mecadox	ZnO	SD
Feed intake N1, lb	12.84^{bc}	12.25^{bc}	11.30^{c}	12.21^{bc}	13.89^{ab}	15.20^{a}	1.86
Feed intake N2, lb	23.34^{bc}	24.75^{abc}	22.69^{c}	20.85^{c}	25.95^{ab}	27.05^{a}	2.92
Total feed intake, lb	36.18^{b}	37.00^{b}	33.98^{b}	33.05^{b}	39.83^{ab}	42.25^{a}	3.99
Daily intake N1, lb/d	0.61^{bc}	0.59^{bc}	0.54^{c}	0.58^{bc}	0.67^{ab}	0.72^{a}	0.09
Daily intake N2, lb/d	1.67^{b}	1.76^{ab}	1.62^{b}	1.49^{b}	1.85^{ab}	1.93^{a}	0.21
Feed/Gain, lb/lb	1.64	1.57	1.53	1.58	1.59	1.59	0.40

^{a,b,c}Treatment effect, $P<0.05$.
BP 1 = BioPlus 2B at 1.1 million spores/gram of feed
BP 2 = BioPlus 2B at 1.3 million spores/gram of feed
$CuSO_4$ = Cooper sulfate
ZnO = Zinc oxide
N1 = Nursery diet 1
N2 = Nursery diet 2

Table 3 – Minimum inhibitory concentrations pre-feeding (Day 0) and post-feeding (Days 21 and 35)

Item	BP 1	BP 2	Control	$CuSO_4$	Mecadox	ZnO	SD
Day 0, µg/ml	2.00^{b}	2.80^{a}	2.00^{b}	2.00^{b}	2.00^{b}	2.00^{b}	0.74
Days 21 and 35, µg/ml	2.00	2.00	2.13^{c}	2.00	2.24	2.00	0.38

^{a,b}Treatment effect, $P<0.05$.
BP 1 = BioPlus 2B at 1.1 million spores/gram of feed
BP 2 = BioPlus 2B at 1.3 million spores/gram of feed
$CuSO_4$ = Cooper sulfate
ZnO = Zinc oxide
N1 = Nursery diet 1
N2 = Nursery diet 2

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