RESEARCH REPORT
PORK SAFETY

Title: Evaluation of antimicrobial alternatives to reduce the development of antibiotic resistance **– NPB #03-075**

Investigator: Darryl Ragland, DVM, PhD

Institution: Purdue University

Date Received: June 16, 2004

Abstract: A 35-day growth assay was conducted to assess the effect of an oregano oil feed additive on nursery pig performance and development of antibiotic resistance in nursery pigs. One hundred eighty pigs, with an average weight of 12.3 pounds and an average of 17 days of age were randomly assigned to three experimental treatments. Experimental treatments consisted of an unmedicated basal diet (Control diet), and diets supplemented with Carbadox at 50 g/ton and an oregano oil extract at 3 pounds/ton. Pigs were weighed weekly to assess growth. Feed added to feeders was recorded and feeder weights were obtained on days 20 and 35 of the study to assess feed consumption. Rectal swabs were collected from pigs on days 0, 20 and 35 of the study for isolation of *E. coli* to determine antimicrobial resistance resulting from consumption of the experimental treatments. Patterns of multiple antimicrobial resistance were similar across dietary treatments at the beginning of the study. Decreases in resistance to several antimicrobials were observed during the course of the study and the respective diets did not encourage progression in the patterns of resistance observed at the beginning of the study. The diet containing the oregano oil extract failed to improve growth rate or feed efficiency in comparison to the Control diet and the diet supplemented with Carbadox supported the best pig performance overall. Based on the conditions of the study, the oregano oil extract evaluated does not represent a viable alternative to feed-grade antimicrobials.

Introduction: The privilege of antimicrobial supplementation of livestock diets has placed animal agriculture at odds with human health interests. Progressive patterns of antibiotic resistance documented in human and zoonotic pathogens worldwide have prompted a challenge to this production practice by human health professionals (Gorbach, 2001). Many in the human health arena have adopted the position that antimicrobials used for growth promotion represents a hazard to human health and this opinion has prompted actions that place the privilege at risk. Lobbying by professional medical organizations and consumer groups have resulted in proposed federal legislation that will hastily phase out antimicrobial supplementation of livestock diets if approved. Restriction of antimicrobial feed additives has been shown to attenuate antibiotic resistance. In Denmark, where antimicrobial growth promoters have been restricted for several years, recent reports indicate that antimicrobial resistance patterns have decreased (Aarestrup et al., 2001). Concomitant with the reported decrease in resistance from restricted antimicrobial supplementation of livestock diets, a significant increase in the therapeutic use of antimicrobials has been reported. Langlois et al. (1988) demonstrated that complete withdrawal of antimicrobial growth promoters from the swine production environment decreases bacterial resistance of fecal coliforms over time. However, resistance to multiple antimicrobials persisted after restriction of growth promoters was instituted. Inasmuch as the study demonstrated that antimicrobial restriction could decrease antimicrobial resistance, it also documented that a stable, resistant microflora from previous antimicrobial exposure survives in near perpetuity.

The literature on oregano oil shows that it has a diversity of potential applications with regards to food processing and food hygiene. Oregano oil has shown the ability to reduce *Salmonella* contamination of refrigerated meats and effectively limit its survival (Skandamis et al., 2002), which has potential application in post-harvest food safety. Oregano oil has been observed to be an effective inhibitor of fungi in stored grain (Paster et al., 1995) and has been described as having parasiticide properties (Force at al., 2000). Numerous studies (Dorman and Deans, 2000; Hammer et al., 1999; Elgayyar et al., 2001) have been conducted to evaluate the antibacterial activity of oregano oil and demonstrate that oregano oil is a potent inhibitor of infectious agents like *Salmonella typhimurium, E. coli,* and *Yersinia enterocolitica*. Two compounds in oregano oil, carvacrol and thymol, are postulated to be responsible for its inhibitory properties, and are considered to act by disrupting cell wall integrity of bacteria (Lambert et al., 2001). While oregano has provided encouraging results in in-vitro studies, the number of performance studies with pigs is minimal and data from these studies has been difficult to access. Feeding studies with pigs have originated predominately from Europe and the results of these studies have been mixed. Van Krimpen et al. (2001) observed enhanced growth in pigs fed oregano oil for only the first 14 days in the nursery, after which, performance did not differ from the unmedicated control. Bilkei and Gertenbach (2001) observed a significant improvement in growth of previously poor growing pigs whose diets were supplemented with oregano oil and vitamin E. It is difficult to formulate an opinion on so few studies, however we consider the described studies significant to this proposal as they demonstrate that oregano oil has growth promoting properties in vivo. Furthermore, we believe that in accordance with appropriate management, sanitation and biosecurity measures, oregano oil may facilitate reductions in antimicrobial use in swine feeding programs without compromising the sustainability of swine production.

In summary, antibiotic supplementation of livestock diets to promote improved performance is being challenged on the grounds that the practice negatively influences public health. Mitigation of antimicrobial resistance requires strategies that promote and

facilitate judicious use of antimicrobials in human medicine and veterinary medicine. Achieving significant reductions in the use of antimicrobials for growth promotion will invariably require the exploitation of antimicrobial alternatives.

Objectives: The overall objective of the study was identification of viable alternatives to antimicrobials for growth promotion 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 antimicrobials in swine feeding programs without sacrificing animal health. Two research objectives were developed. The first research objective was assessment of the ability of oregano essential oil to alter antimicrobial resistance patterns. The mechanism that enables oregano oil to inhibit bacterial growth is postulated to be disruption of bacterial cell wall integrity. The mechanism of cell wall disruption is common to numerous antimicrobials, and some bacteria have developed the ability to resist this mechanism of action. The working hypothesis for this objective is while oregano oil shares a common mechanism of inhibition with several antimicrobials, its suitability as an antimicrobial alternative is dependent on not promoting development of antimicrobial resistance. The second research objective is assessment of animal health and performance in response to dietary supplementation of oregano oil. The ability to suppress disease and support acceptable levels of performance in nursery pigs represents recognized properties of antimicrobial feed additives. The working hypothesis for this objective is the value of oregano oil in swine feeding should be judged based on its capacity to enhance pig performance and minimize disease in the critical nursery phase of growth.

Procedures: Objective 1. A 35-day growth assay was employed to assess the effect of oregano oil on the development of antimicrobial resistance. A randomized complete block experimental design was used with 10 replicate blocks. The pen served as the experimental unit. Three dietary treatments were formulated. Diets consisted of an unmedicated basal diet (Control) and a medicated diet. Carbadox was added to the basal diet at a rate of 50 g/ton of feed. A commercial oregano oil extract manufactured by Van Beek Scientific was added to the basal diet at a rate of 3 pounds/ton of feed. One hundred eighty crossbred pigs, with an average weaning age of 17 days were weighed, sorted according to weight, assigned to treatments and placed in the nursery. The pigs were obtained from a commercial swine farm that practiced early weaning and were considered to be high health status pigs. As part of the farm's sow management, the lactation diet was supplemented with CSP 250. Experimental treatments were assigned to 30 pens and six pigs were housed in each pen. *Escherichia coli (E. coli)* was cultured from pigs and served as the sentinel organism to ascertain the level of antimicrobial resistance associated with the experimental treatments. Rectal swabs containing Stuart's transport medium were collected from three pigs in each pen on days 0, 20 and 35 of the study and cultured on MacConkey agar. Colonies were collected from the MacConkey plates and plated onto blood agar to obtain pure cultures for gram staining identification and susceptibility testing. The agar dilution susceptibility assay was used to determine antimicrobial resistance of the fecal isolates. The isolates were screened for resistance using the antibiotic panel employed by the National Antimicrobial Resistance Monitoring System (NARMS) which consists of amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, kanamycin, nalidixic acid,

streptomycin, sulfasoxazole, tetracycline and trimethoprim/sulfasoxazole. Concentrations of antimicrobials used in the microdilution assay to screen the isolates for resistance were 0, 0.5, 1, 2, 3, 4, 8, 16, 32, 64, 128 and 256 µg/ml. In preparation of the combination antimicrobials, amoxicillin was incorporated with clavulanic acid at a 4:1 ratio and sulfasoxazole was incorporated with trimethoprim at a 5:1 ratio. 96-well plates were prepared, inoculated and incubated at 35^0C and evaluated after 24 hours of incubation of all plates. Least square means for minimum inhibitory concentrations (MIC) were calculated and subjected to the GLM procedure of SAS®. The least significant difference test was used as the mean separation procedure.

Objective 2. Objective 2 of the proposed study was performed concurrently during the completion of Objective 1. To assess performance, pigs enrolled in the study were weighed weekly after the beginning of the study to evaluate growth and calculate average daily gain. Feeder weights were obtained on days 20 and 35 to calculate feed intake by pen and calculate feed efficiency. 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 performance aspect of the study. Least square means for performance (weight, average daily gain, feed intake, feed efficiency) 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. Minimum inhibitory concentration results are shown in Table 1. On days 0, 20 and 35 of the study, all *E. coli* isolates were sensitive to amikacin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and trimethoprim/sulfasoxazole based on their MIC breakpoints. Isolates were resistant to amoxicillin/clavulanic acid, ampicillin, cephalothin, florfenicol, streptomycin, sulfasoxazole and tetracycline on Day 0. Moreover on day 0, *E. coli* from pigs assigned the Control and oregano diets were resistant to apramycin and isolates from pigs fed the Carbadox diet were resistant to kanamycin. *E. coli* continued to exhibit resistance to amoxicillin/clavulanic acid, ampicillin, florfenicol, sulfasoxazole and tetracycline when measured on days 20 and 35 of the study. On day 20 of the study, isolates became susceptible to apramycin, kanamycin and streptomycin. Isolates from pigs assigned the oregano diet continued to exhibit resistance to cephalothin. On day 35 of the study, all isolates remained susceptible to streptomycin and exhibited resistance to cephalothin. Isolates from pigs assigned the Carbadox diet were resistant to apramycin and isolates from pigs assigned the Control diet were resistant to kanamycin.

Objective 2. Growth, average daily gain, feed intake and feed efficiency data are shown in Table 2. Pigs fed the Carbadox diet achieved the heaviest bodyweights, highest average daily gain, highest feed intake and greatest feed efficiency ($P>0.05$). The Control diet yielded the second best pig performance and exceeded the performance of pigs fed the diet supplemented with the oregano oil extract. The reduced feed intake of the pigs fed the diet supplemented with oregano oil was most likely a function of feed refusal related to palatability of the diet. Addition of the oregano oil feed additive made the diets strongly aromatic. Oregano is a member of the mint family and is described as having a pungent odor and taste. The mint odor of the dehydrated product was very apparent prior to mixing in the diet. After mixing, the odor of the product was still

apparent but not as pronounced as the dehydrated product. This assumed to have exerted a negative effect on feed intake and subsequent pig growth.

Discussion: Objective 1. The level of multiple antimicrobial resistance was noteworthy at the beginning of the study as the *E. coli* isolates showed resistance to 9 of 17 antimicrobials on the panel. The high level of resistance observed for amoxicillin/clavulanic acid, ampicillin, sulfasoxazole and tetracycline were likely related to the fact that the sow lactation diet was supplemented with CSP 250, a feed-grade antimicrobial consisting of chlortetracycline, sulfathiazole and penicillin. This would allow introduction of resistant bacteria into the nursing pig environment via the sows and enable their exposure to resistant bacteria shed in sow feces. Changes in the level of antimicrobial resistance became apparent during the course of the study. Reductions in MIC for amoxicillin/clavulanic acid, ampicillin and streptomycin occurred across all dietary treatments and variations in sensitivity were apparent for apramycin, cephalothin, kanamycin and trimethoprim/sulfasoxazole. Overall, the MIC data indicate that Carbadox did not promote resistance that varied in magnitude to the Control and oregano diets. Therefore, no apparent benefit to using the oregano oil feed additive can be appreciated based on the results of the MIC determinations.
Objective 2. The performance data clearly demonstrate that the oregano oil feed additive was inferior to Carbadox at promoting growth and enhancing feed utilization. The reduced feed intake compared to the Control diet suggests that the palatability issue is probably responsible for the poor performance of pigs fed the diet supplemented with oregano oil. The 3 pounds/ton rate of inclusion was the maximum recommended level. Speculatively, a lower level of inclusion may have promoted better feed intake and been more tolerable to the pigs. More research is warranted to determine if palatability of the diet was the major factor that limited intake of the pigs fed oregano oil extract.

Lay interpretation: The oregano oil product evaluated did not enhance nursery pig performance or influence the development of antimicrobial resistance to the panel of antibiotics used to screen resistance. While a high level of multiple antimicrobial resistance was apparent at the beginning of the study, decreases in the level of resistance to several of the antibiotics was an attribute of all the dietary treatments. Therefore, the oregano oil product demonstrated no apparent benefit over Carbadox or the Control diet in modulating antimicrobial resistance. The less than superb performance obtained from feeding the oregano oil product appears to be a function of depressed feed intake based on the intake and feed efficiency data. The strongly aromatic nature of the oregano oil product most likely discouraged consumption of the diet resulting in the poor pig performance.

Table 1 - Mean Resistance of *E. coli*
Minimum Inhibitory Concentrations

Day 0 MIC (µg/mL)

Diet	Amik	Amox	Ampi	Apra	Ceftri	Ceftri	Ceph	Chlor	Cipro	Flor	Gent	Kana	Nali	Strept	Sulfa	Tet	TMS
Control	0.85[a]	198.96[b]	205.33[b]	55.58[a]	0.50[b]	0.50	56.00[a]	16.83	0.50	53.33[a]	1.52[b]	33.19[b]	2.17[b]	88.25[a]	256.00[a]	256.00	11.44[b]
Carbadox	0.74[b]	170.72[c]	190.40[c]	2.40[c]	0.56[a]	0.50	55.16[a]	19.04	0.50	50.56[a]	0.50[c]	92.70[a]	2.16[b]	81.84[a]	250.88[b]	256.00	21.28[a]
Oregano	0.69[c]	213.81[a]	221.33[a]	38.33[b]	0.50[b]	0.50	52.19[a]	17.05	0.50	54.10[a]	1.95[a]	37.57[b]	2.45[a]	86.24[a]	256.00[a]	256.00	1.00[c]
SE	0.04	12.39	11.90	9.46	0.01	0	4.11	2.94	0	2.78	0.30	12.32	0.11	7.68	1.83	0	6.23
Resistance Breakpoint	>64	>32/16	>32	>32	>8	>64	>32	>32	>4	>16	>16	>64	>32	>64		>16	>4/76

[abc]Superscripts in columns denote differences at P<0.05

Day 20 MIC (µg/mL)

Diet	Amik	Amox	Ampi	Apra	Ceftri	Ceftri	Ceph	Chlor	Cipro	Flor	Gent	Kana	Nali	Strept	Sulfa	Tet	TMS
Control	0.62	87.38[c]	103.43[c]	1.88[b]	0.50	0.50	25.90[b]	7.95[b]	0.50	48.76[b]	0.50	58.52[a]	2.14[b]	51.57[a]	217.90[b]	256.00	0.60[c]
Carbadox	0.67	113.96[b]	169.38[b]	11.44[a]	0.50	0.50	21.85[c]	8.54[ab]	0.50	45.54[c]	0.50	33.87[c]	3.55[a]	24.61[b]	214.15[b]	256.00	10.44[b]
Oregano	0.63	192.58[a]	205.00[a]	1.58[b]	0.50	0.50	32.75[a]	9.17[a]	0.50	52.00[a]	0.50	41.81[b]	1.96[b]	60.88[a]	235.33[a]	256.00	22.00[a]
SE	0.05	12.94	13.71	3.59	0	0	2.17	1.17	0	1.91	0	7.28	0.47	9.41	8.89	0	6.15
Resistance Breakpoint	>64	>32/16	>32	>32	>8	>64	>32	>32	>4	>16	>16	>64	>32	>64		>16	>4/76

[abc]Superscripts in columns denote differences at P<0.05

Day 35 MIC (µg/mL)

Diet	Amik	Amox	Ampi	Apra	Ceftri	Ceftri	Ceph	Chlor	Cipro	Flor	Gent	Kana	Nali	Strept	Sulfa	Tet	TMS
Control	0.91[a]	73.94[c]	98.88[c]	1.72[c]	0.50	0.50	34.00[b]	14.00[b]	0.50	60.44[a]	0.52[c]	69.86[a]	2.75[a]	45.00[a]	256.00[a]	256.00[a]	0.94[b]
Carbadox	0.79	166.5	162.6	75.9	0.50	0.50	43.00	21.00	0.50	56.00	2.06	20.35[c]	2.63	40.54	234.6	236.0	2.19

	[b]	4[a]	7[b]	0[a]			[a]	[a]		[b]	[a]		[a]	[a]	6[c]	0[b]	[b]
Oregano	0.65[c]	135.80[b]	166.74[a]	14.43[b]	0.50	0.50	36.40[b]	24.40[a]	0.50	54.40[b]	0.88[b]	40.60[b]	2.15[b]	17.45[b]	244.00[b]	232.00[b]	13.45[a]
SE	0.08	14.81	16.50	10.22	0	0	3.97	3.60	0	2.82	0.30	9.12	0.27	6.63	6.05	7.57	4.13
Resistance Breakpoint	>64	>32/16	>32	>32	>8	>64	>32	>32	>4	>16	>16	>64	>32	>64		>16	>4/76

[abc]Superscripts in columns denote differences at P<0.05

Abbreviation legend

Amik = Amikacin
Amox = Amoxicillin/ clavulanic acid
Ampi = Ampicillin
Apra = Apramycin
Cefti = Ceftiofur
Ceftri = Ceftriaxone
Ceph = Cephalothin
Chlor = Chloramphenicol
Cipro = Ciprofloxacin
Flor = Florfenicol
Gent = Gentamicin
Kana = Kanamycin
Nali = Nalidixic acid
Strept = Streptomycin
Sulfa = Sulfasoxazole
Tet = Tetracycline
TMS = Trimethoprim/Sulfasoxazole

Table 2 – Bodyweight, average daily gain, feed consumption and feed efficiency

Item	Control	Carbadox	Oregano	SE
Day 0 weight, lb	12.3	12.3	12.3	0.09
Day 20 weight, lb	25.6[b]	26.3[a]	24.4[c]	0.22
Day 35 weight, lb	43.5[b]	45.3[a]	41.6[c]	0.31
ADG Day 0-35, lb/d	0.89[b]	0.94[a]	0.84[c]	0.008
Total feed intake, lb	311.22[b]	320.79[a]	289.19[c]	3.22
Feed:Gain, lb/lb	1.66[b]	1.62[c]	1.69[a]	0.01

[abc]Superscripts in rows denote differences at P<0.05

ADG = Average daily gain

References cited

Gorbach SL. Antimicrobial use in animal feed – time to stop. New England Journal of Medicine. 2001;345:1202-1203.

Aarestrup FM, Seyfarth AM, Emborg H-D, Pederson K, Hendriksen RS, and Bager F. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrobial Agents and Chemotherapy. 2001;45:2054-2059.

Langlois BE, Dawson KA, Leak I, and Aaron DK. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. Veterinary Microbiology. 1988;18:147-153.

Skandamis PN, Tsigarida E, and Nychas G-JE. The effect of oregano essential oil on survival/death of Salmonella typhimurium in meat stored at 5^0C under aerobic, VP/MAP conditions. Food Microbiology. 2002;19:97-103.

Paster N, Menasherov M, Ravid U, and Juven B. Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. Journal of Food Protection. 1995;58:81-85.

Force M, Sparks WS, and Ronzio RA. Inhibition of enteric parasites by emulsified oil of oregano in vivo. Phytotherapy Research. 2000;14:213-214.

Dorman HJD and Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology. 2000;88:308-316.

Hammer KA, Carson CF, and Riley TV. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology. 1999;86:985-990.

Elgayyar M, Draughon FA, Golden DA, and Mount JR. Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. Journal of Food Protection. 2001;64:1019-1024.

Lambert RJW, Skandamis PN, Coote PJ, and Nychas G-JE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology. 2001;91:453-462.

van Krimpen MM and Binnendijk GP. Ropadiar as alternative for antimicrobial growth promoter in diets of weanling pigs. Rapport Praktijkonderzoek Veehouderij. 2001;205.

Bilkei G and Gertenbach W. Retrospective evaluation of the combined effect of high vitamin E and oregano phytogenic feed additives on the performance of “slow growing” fattening pigs. Biologische Tiermedizin. 2001;18:83-87.