Effects of quaternary benzo(c)phenanthridine alkaloids on growth performance, shedding of organisms, and gastrointestinal tract integrity in pigs inoculated with multidrug-resistant *Salmonella* spp

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Objective—To evaluate effects of quaternary benzo(c)phenanthridine alkaloids (QBAs) against *Salmonella* spp and determine effects on growth performance, organism shedding, and gastrointestinal tract integrity in pigs inoculated with *Salmonella enterica* serovar Typhimurium.

Sample—36 *Salmonella* isolates and twenty 5-week-old pigs.

Procedures—Minimum inhibitory concentration of QBAs against the *Salmonella* isolates was determined. Pigs were allocated to 4 groups and inoculated with *Salmonella* organisms. Pigs received diets supplemented with 1.5 g of QBAs/1,000 kg of feed, 0.75 g of QBAs/1,000 kg of feed, or 59.4 g of chlortetracycline/1,000 kg of feed or a nonsupplemented (control) diet. Pigs were weighed on day 0 and then weekly for 40 days. Fecal samples were collected to quantify *Salmonella* organisms. Gastrointestinal tract integrity was evaluated by measuring transepithelial resistance.

Results—In vitro, 9 of 36 (25%) *Salmonella* isolates were inhibited at 90 µg of QBAs/mL; all 36 were inhibited at 179 µg of QBAs/mL. Diets containing QBAs significantly decreased *Salmonella* spp shedding; shedding was lower 40 days after inoculation for pigs fed diets containing QBAs or chlortetracycline than for pigs fed the control diet. Growth performance was similar for pigs fed diets containing QBA or chlortetracycline. Gastrointestinal tract integrity was improved in pigs fed the diet containing 1.5 g of QBAs/1,000 kg of feed.

Conclusions and Clinical Relevance—QBAs and chlortetracycline decreased *Salmonella* spp shedding but did not differ with regard to growth performance. Gastrointestinal tract integrity was better, albeit not significantly, in pigs fed diets containing QBAs. Further investigation into the role of QBAs and their mechanism as an immunomodulator is necessary. (*Am J Vet Res* 2013;74:1530–1535)

Salmonella enterica is a ubiquitous enteric pathogen estimated to cause > 1.4 million cases of illness in humans in the United States annually,[1] of which 95% are estimated to result from foodborne transmission.[2] In addition, the increased incidence of multidrug-resistant infections caused by phage types such as *S Enterica* serovar Typhimurium DT104[3] and the isolation of this strain from swine[4] may pose serious health risks to pork consumers.

Abbreviations

ADG	Average daily gain
DT	Determinant type
MPN	Most probable number
QBA	Quaternary benzo(c)phenanthridine alkaloid
TER	Transepithelial resistance

The addition of antimicrobials to swine diets is a common practice in most parts of the world, including the United States.[5] Chlortetracycline is estimated to be the antimicrobial most widely used in feed for the nursery and growth and finishing phases of pork production.[6] Feeding of antimicrobials, including chlortetracycline, for growth promotion can decrease fecal shedding of enteric pathogens[7] and could serve to improve preharvest food safety by minimizing carcass contamination during processing.

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Because of increasing concerns surrounding use of antimicrobials at subtherapeutic concentrations in food animals, the pork industry has funded investigations on alternatives to traditional antimicrobials used as growth or production promoters; those alternatives include herbs, immune modulators, and probiotics. Limited investigations have been conducted on the use of herbal extracts, particularly those containing isoquinolone alkaloids, for their antimicrobial properties.[8] An isoquinolone alkaloid, QBA, reportedly has anti-inflammatory and antimicrobial properties[9–12] and can decrease amino acid degradation, increase feed intake, and promote growth in swine.[13,14] In 1 study,[15] QBAs significantly decreased damage to the colonic mucosa and mitigated colonic inflammation when included in the feed of rats, which suggests a protective effect of QBAs on the intestinal mucosa. Quaternary benzo(c) phenanthridine alkaloid consists of sanguinarine and chelerythrine extracts. Neither QBAs nor any proprietary formulations have been evaluated for their effects on the antibiogram of foodborne pathogens, the ability to decrease bacterial fecal shedding, or improvement in intestinal wall barrier function. Thus, the purpose of the study reported here was to evaluate the in vitro effects of QBAs on *Salmonella* isolates and compare the effects of QBAs with those of chlortetracycline on growth performance, feed efficiency, and fecal shedding of *Salmonella* organisms in nursery-age pigs. Our hypothesis was that *Salmonella* spp shedding is influenced by feed-grade antimicrobials and that a nonantimicrobial alternative (QBA) could serve as a potential replacement to traditional antimicrobials.

Materials and Methods

Animals—Twenty 5-week-old crossbred gilts (mean ± SD body weight, 8.96 ± 0.26 kg) with negative results for culture of *Salmonella* spp in feces were used in the study. Only pigs that had received ceftiofur[a] at the labeled dose and had 2 consecutive negative results for culture of *Salmonella* spp in fecal samples obtained on the day of ceftiofur treatment and 5 days after ceftiofur treatment were enrolled.

Pigs were housed individually at 26 ± 2°C in pens (3.05 × 0.76 × 1.37 m); there was a solid barrier between pens to prevent direct contact. Pigs were fed a 2-phase mash diet. The phase 1 diet (45% corn, 29% soybean meal, and 19.98% dried whey) was fed for 14 days. The phase 2 diet (62.5% corn and 30% soybean meal) then was fed for the remainder of the 40-day study. Diets were formulated on the basis of nutritional guidelines to meet or exceed dietary requirements for growing pigs.[16] Feed and water were provided ad libitum throughout the study. Animal care was provided in accordance with a protocol approved by the North Carolina State University Animal Care and Use Committee.

In vitro assay—Inhibitory effects of QBAs on various strains of *Salmonella* spp were evaluated by use of the agar dilution method in accordance with standard reference methods recommended by the Clinical and Laboratory Standards Institute. Briefly, QBAs obtained from *Macleaya cordata* extract were diluted in sterilized deionized water to a concentration of 33 g/L and mixed with Müeller-Hinton agar[b] at concentrations ranging from 0 to 896 µg/mL. Thirty-six strains previously isolated from swine, including *S enterica* serovars Typhimurium, Heidelberg, and Derby, that represented pansusceptible and multidrug-resistant strains were tested. Spots of a suspension of fresh inoculum were placed on the agar with a replicator system.[c] A control plate with no QBAs was also cultured. Plates were incubated for 24 hours at 37°C and visually evaluated for growth.

Experimental design—Pigs were allocated by use of a randomization procedure (ie, random numbers generated with software[d]) to a room, a pen, and 1 of 4 experimental diets (5 pigs/treatment). Pigs were fed diets supplemented with 1.5 g of QBAs/1,000 kg of feed, 0.75 g of QBAs/1,000 kg of feed, or 59.4 g of chlortetracycline/1,000 kg of feed or a nonsupplemented control diet. Dietary concentrations of QBAs[e] and chlortetracycline[f] used in the study were fed as per label directions to improve rate of weight gain and feed efficiency. Pigs were challenge-inoculated with *Salmonella* organisms on day 0 and euthanized (xylazine and pentobarbital; doses determined on the basis of body weight) on day 40. However, 1 pig fed the control diet and 1 pig fed the diet containing 0.75 g of QBAs/1,000 kg of feed were euthanized on days 6 and 13, respectively, because of conditions unrelated to the study.

Challenge inoculation with *Salmonella* organisms—*Salmonella* Typhimurium DT104 with a penta-resistant (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) profile was used as the challenge strain. This isolate had been previously isolated from swine feces during the in vitro assay. The inoculum was grown to mid-logarithmic phase in Luria-Bertani broth[b] with agitation at 37°C. On day 0, each pig received via oral drench 5 mL of inoculum containing 1.0×10^8 CFUs (as determined by enumeration on Müeller-Hinton agar[b]). This dose was consistent with doses used in another study.[17]

***Salmonella* isolation, antimicrobial susceptibility testing, and quantification**—Fecal samples were collected from each pig on days 2, 6, 12, 19, 26, 33, and 40. *Salmonella* organisms were isolated with tetrathionate broth[b] enrichment as described elsewhere.[18] The antimicrobial resistance patterns for up to 5 *Salmonella* isolates were determined with commercially available antimicrobial susceptibility plates[g] to confirm whether the challenge strain was the same as the strain cultured from the fecal samples. All isolates were evaluated for antimicrobial susceptibility with the Kirby-Bauer disk diffusion test. Quantification was conducted in accordance with the 3 dilution × 3 tube MPN method and calculated with an MPN calculator.[h] *Salmonella* organism counts that were estimated as < 1 CFU/g of feces by the MPN method were grouped into a single category for statistical analysis.

TER—Transepithelial resistance is a sensitive measure of intestinal barrier function and reflects the ability of epithelium to impart a resistance barrier (governed by integrity of the interepithelial tight junctions) across the epithelium. Measurement of TER was conducted

in an Ussing chamber as described elsewhere.[19] Briefly, fresh intestinal samples were collected from each pig immediately after the pigs were euthanized on day 40. Intestinal samples (sections of ileal tissue) were collected and mounted on an Ussing chamber. Mucosal barrier function was determined by measuring the electrical resistance per area of the intestine. Measurements were recorded as a continuous variable ranging between 0 and 80 Ω/cm^2, and TER values were compared between uninfected control pigs (2 pigs that originated from the same source farm and had the same genetic background as the other pigs and were tested and found to be negative for *Salmonella* spp) and *Salmonella*-challenged pigs receiving chlortetracycline or 1.5 g of QBAs/1,000 kg of feed. A TER of 40 Ω/cm^2 is considered typical for a clinically normal pig.

Neutrophil counts were determined as a measure of overall intestinal health and to confirm findings from the TER assay. Ileal tissues were fixed in formalin and stained with H&E. The extent of neutrophil infiltration was measured qualitatively with microscopic evaluation and quantified as the number of neutrophils per square millimeter.

Assessment of ADG and feed conversion ratio— Consumption of feed was calculated as weight of feed offered minus weight of unconsumed feed and reported as mean daily feed intake on a dry-matter basis. Pigs were weighed on days 0, 7, 14, 21, 28, 35, and 40 for calculation of weight gain, ADG, and the feed conversion ratio (ie, ratio of feed consumption to weight gain). Data for the 2 pigs euthanized prior to day 40 were not included in the statistical analysis for these variables.

Statistical analysis—The use of 1-tailed or nondirectional tests increases statistical power to detect a difference in the expected direction.[20] Investigators in other studies[21–23] have found that inclusion of antimicrobials in diets of nursery pigs improves growth performance. Therefore, the expected outcome that chlortetracycline would improve growth and the feed conversion ratio of nursery pigs challenge-inoculated with *Salmonella* Typhimurium DT104 was assessed with a 1-tailed analysis. Pairwise comparisons of ADG, feed conversion ratio, and fecal shedding of *Salmonella* organisms were performed for pigs receiving diets supplemented with QBAs or chlortetracycline versus those for pigs receiving the control diet, pigs receiving diets supplemented with QBAs versus pigs receiving diets supplemented with chlortetracycline, and pigs receiving the diet supplemented with 1.5 g of QBAs/1,000 kg of feed versus pigs receiving the diet supplemented with 0.75 g of QBAs/1,000 kg of feed. Comparisons were conducted by means of a Wilcoxon rank sums test with standard statistical software.[i] Spearman correlation coefficients for fecal shedding of *Salmonella* organisms within a treatment group were calculated with standard statistical software.[i] Correlations between fecal shedding of *Salmonella* organisms and time after inoculation for each treatment group were plotted with commercial graphing software[j] and examined. Significance was determined on the basis of 1-sided tests; values were considered significant at $P < 0.05$.

Results

In vitro assay—The in vitro inhibitory effect of QBAs on *Salmonella* isolates was determined (**Table 1**). Of 36 *Salmonella* isolates, 9 (25%) were inhibited at 90 µg of QBAs/mL; the remaining 27 isolates were able to grow at that concentration of QBAs. However, all 36 *Salmonella* isolates were inhibited at a concentration of 179 µg of QBAs/mL.

ADG and feed conversion ratio—Weight gain and feed efficiency were measured as ADG and the feed conversion ratio (**Table 2**). Pigs receiving diets supplemented with QBAs or chlortetracycline did not have a significantly better ADG or feed conversion ra-

Table 1—Minimum inhibitory concentration of QBAs for 36 *Salmonella enterica* isolates from swine.

Serovar	Phage type of isolates	Total No.	No. of isolates inhibited: 90 µg of QBAs/mL	No. of isolates inhibited: 179 µg of QBAs/mL
Typhimurium	DT104	7	3	7
	DT193	11	1	11
	DT208	4	0	4
	DT21	1	1	1
	NA	8	2	8
Derby	NA	3	1	3
Heidelberg	NA	2	1	2

NA = Not applicable.

Table 2—Mean ± SD body weight, mean daily feed intake, ADG, and feed conversion ratio for 18 pigs fed various experimental diets and inoculated via oral drench with *Salmonella enterica* serovar Typhimurium DT104.

Diet	Body weight (kg): Day 0	Body weight (kg): Day 40	Mean daily feed intake (kg)	ADG (kg)	Feed conversion ratio
1.5 g of QBAs/1,000 kg of feed (n = 5)	8.58 ± 0.58	29.40 ± 3.38	1.00 ± 0.04	0.52 ± 0.08	1.94 ± 0.23
0.75 g of QBAs/1,000 kg of feed (n = 4)	9.19 ± 0.57	31.25 ± 2.06	0.80 ± 0.43	0.55 ± 0.06	1.81 ± 0.19
59.4 g of chlortetracycline/1,000 kg of feed (n = 5)	9.75 ± 0.76	31.70 ± 2.08	0.98 ± 0.07	0.55 ± 0.03	1.80 ± 0.09
Unsupplemented control (n = 4)	8.98 ± 1.00	28.50 ± 3.50	0.83 ± 0.46	0.49 ± 0.09	2.18 ± 0.44

Day of inoculation was designated as day 0. Feed was weighed daily, and pigs were weighed weekly. No significant ($P \geq 0.05$) differences in growth performance variables were detected among treatment groups.

tio, compared with results for pigs receiving the nonsupplemented control diet. In addition, there was no significant improvement in ADG and feed conversion ratio for pigs receiving diets supplemented with QBAs (1.5 or 0.75 g/1,000 kg of feed), compared with results for pigs receiving the diet supplemented with chlortetracycline. The overall ADG and feed conversion ratio were within expected ranges for growing pigs,[16] despite challenge inoculation with *Salmonella* organisms.

Effect of diet on fecal shedding of *Salmonella* organisms was determined by measuring the number of CFUs per gram of feces obtained from each pig during the 40-day study. We assumed that the *Salmonella* colonies were indicative of fecal shedding of *Salmonella* Typhimurium DT104, given that pigs had negative results prior to challenge inoculation; no isolates were serotyped or phage typed after isolation. The distribution of pigs receiving a supplemented diet, whether with chlortetracycline or either concentration of QBA, was significantly different from the distribution of those fed the control diet at days 26 and 40. Pigs receiving supplemented diets shed *Salmonella* organisms at a median of 9.3 CFUs/g of feces on day 26 and in the range of 2.3 to 2.8 CFUs/g of feces on day 40, compared with a median of 110 CFUs/g of feces on day 26 and a range of 24 to 46 CFUs/g of feces on day 40 for those receiving the control diet. In addition, the median number of *Salmonella* organisms shed on day 40 by pigs receiving QBAs in the diet was < 1 CFU/g of feces, which was significantly lower than that shed by pigs receiving chlortetracycline (median, 9.3 CFUs/g of feces). There was no significant difference in fecal shedding of *Salmonella* organisms between pigs receiving 1.5 g of QBAs/1,000 kg of feed or 0.75 g of QBAs/1,000 kg of feed at any time after inoculation.

Strong negative correlations were found between fecal shedding of *Salmonella* organisms and time after inoculation for each of the treatment groups (**Figure 1**). In each group, the time after inoculation accounted for > 25% of the variation in MPN of *Salmonella* organisms shed in feces (data not shown). Although the correlation for all treatment groups was $r \leq -0.50$, the strongest linear relationship ($r = -0.81$) was for the pigs fed 0.75 g of QBAs/1,000 kg of feed. Diets containing 1.5 g of QBAs/1,000 kg of feed, 0.75 g of QBAs/1,000 kg of feed, or 59.4 g of chlortetracycline/1,000 kg of feed significantly decreased the number and expected duration of shedding of *Salmonella* organisms as determined on the basis of the inverse relationship. Although there was a decrease in the shedding of *Salmonella* organisms for the QBAs- and chlortetracycline-treated pigs, compared with that in pigs fed the control diet, the overall differences were small.

Ileal samples from pigs fed the diet supplemented with 1.5 g of QBAs/1,000 kg of feed had a higher mean TER (62 Ω/cm^2), which indicated enhanced health of the mucosal barrier, compared with the mean TER for pigs fed the diet supplemented with chlortetracycline

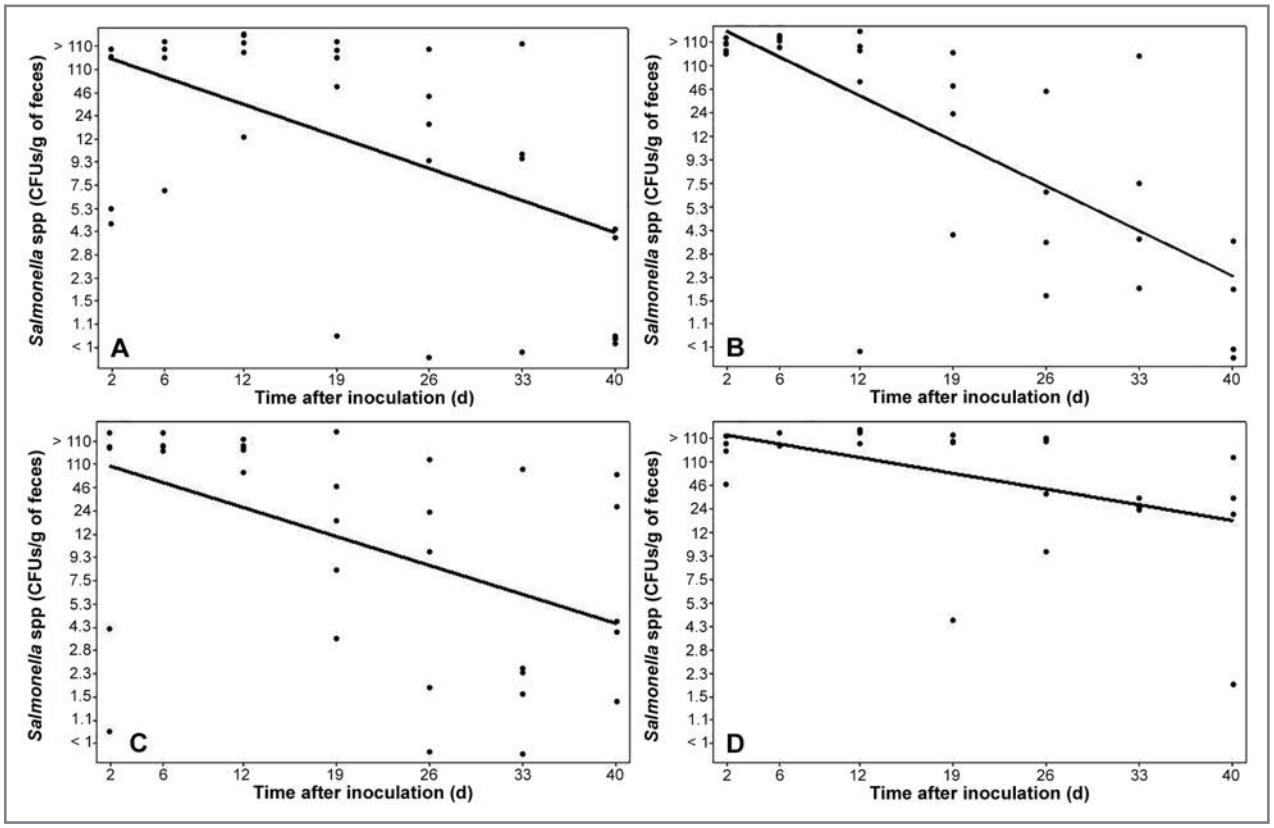

Figure 1—Fecal shedding of *Salmonella* organisms (determined as the MPN) for growing pigs fed diets supplemented with 1.5 g of QBAs/1,000 kg of feed (A [n = 5]), 0.75 g of QBAs/1,000 kg of feed (B [4]), or 59.4 g of chlortetracycline/1,000 kg of feed (C [5]) or fed an unsupplemented control diet (D [5]) and inoculated via oral drench with *Salmonella enterica* serovar Typhimurium DT104. Day of inoculation was designated as day 0. For each panel, the solid line represents the correlation for the data.

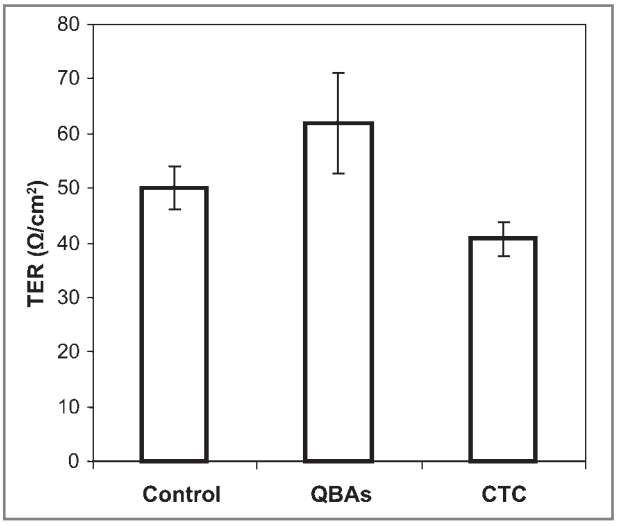

Figure 2—Mean ± SD TER for ileal samples obtained on day 40 from control pigs from the same farm and with the same genetic background and that had negative results when tested for *Salmonella* spp (n = 2) and pigs fed a diet supplemented with 1.5 g of QBAs/1,000 kg of feed (5) or a diet supplemented with 59.4 g of chlortetracycline/ton of feed (5). CTC = Chlortetracycline.

(41 Ω/cm²) or uninfected control pigs (2 pigs that originated from the same source farm and had the same genetic background as the other pigs and were tested and found to be negative for *Salmonella* spp [50 Ω/cm²]; **Figure 2**). This measurement was conducted on samples obtained at a single time point (day 40) and therefore did not provide conclusive results as to whether dietary supplementation with QBAs was protective or resulted in enhanced repair of barrier function following challenge inoculation with *Salmonella* organisms. There were no differences in results of histologic evaluation of the ileal samples among treatments, as determined on the basis of qualitative and quantitative neutrophil counts (results not shown).

Discussion

One of the objectives of the present study was to evaluate the in vitro effect of QBAs on *Salmonella* spp growth in cultures. The QBAs at a concentration of 90 µg/mL inhibited the growth of 3 serotypes and 4 phage types of *Salmonella* spp. These results agree with those of other studies[7,24,25] in which QBAs were found to have antimicrobial effects, but to our knowledge, this is the first report of the minimum inhibitory concentrations of QBAs for *Salmonella* Typhimurium, Heidelberg, and Derby. Nevertheless, concentrations of QBAs used in the in vitro experiments were higher than concentrations typically included in feed, and more studies that involve the use of feed-grade antimicrobials at recommended concentrations are needed to determine the antimicrobial effects of QBAs in field settings.

The absence of a significant increase in ADG or decrease in feed conversion ratio for pigs receiving diets supplemented with QBAs or chlortetracycline in the study reported here may have been associated with the optimum housing and management conditions of pigs in a university research facility. Investigators in other studies[21,22] reported that the true effects of growth promoters, especially those with antimicrobial properties, often are not realized in a research setting because of the cleanliness and strict biosecurity maintained in these facilities. Specifically, production performance for growing pigs raised on commercial farms has been reported to be up to 2 times as great as for growing pigs raised in research facilities when antimicrobials are included in diets at subtherapeutic concentrations.[19] Pigs were not commingled or challenge-inoculated with multiple pathogens, so the effect of diet on growth performance may have been obscured. The authors also recognize that the effects reported in this study, or lack thereof, could have been attributable to random errors that may have been caused by the low sample size.

Growth promotants have cumulative benefits on pig performance and health.[21,22] It has been estimated that 80% of diets formulated for growing pigs and 50% of finishing diets formulated for pigs contain at least 1 antimicrobial at a subtherapeutic concentration that is meant to increase growth and feed efficiency.[21] In light of the historical benefits of antimicrobials as growth promotants as well as the current interest in identifying nonantimicrobial alternatives, we believed it was pertinent to compare the growth performance benefits attributable to a traditional feed-grade growth promotant (chlortetracycline) with benefits for a novel nonantimicrobial alternative (QBAs). This study revealed that benefits to pig growth and feed efficiency were similar regardless of the type of growth promotant administered. Further evaluation of pigs fed diets containing QBAs throughout the postweaning period may also reveal positive benefits, compared with benefits for diets containing chlortetracycline, on growth performance that could not be measured or were not significantly different in the present study.

The plant-derived alkaloids evaluated in this study were more effective than chlortetracycline for decreasing fecal shedding of *Salmonella* organisms at 40 days after inoculation. In addition, fecal shedding in pigs fed diets containing 1.5 g of QBAs/1,000 kg of feed and 0.75 g of QBAs/1,000 kg of feed was strongly correlated with time after inoculation (Figure 1), which suggested that the use of QBAs had an inverse relationship with fecal shedding of *Salmonella* spp after inoculation. The accuracy of the procedure for enumeration of *Salmonella* organisms (MPN calculation) was likely to have affected the analysis. Although this method is widely regarded as a standard microbiological procedure for quantification of bacteria cultured from food products, it does not appear to be appropriate for enumeration of large numbers of bacteria, considering that the highest value represented was 110 CFUs/g and that no higher numeric estimate could be obtained. Nonetheless, on the basis of these observations, QBAs appear to have an application as an in-feed intervention strategy to reduce the chance of antemortem, perimortem, and postmortem contamination in pig rearing and slaughter facilities that results from pigs shedding high numbers of *Salmonella* organisms in the feces.

Certain pathological events, which include intestinal injury, enteric disease (eg, salmonellosis), and stress, initiate the breakdown of intestinal barrier function, which is highlighted by increased gastrointestinal tract permeability as measured by the TER. Leaky in-

testines, indicated by a low TER, allow luminal agents such as bacteria, toxins, or antigens to freely traverse the intestinal epithelium and gain access to subepithelial tissues, which results in inflammation.[26] Once bacteria, toxins, or antigens breach the subepithelium, they potentially gain access to the systemic circulation, which can lead to septicemia, a sequela to multiple organ disease. In the present study, the TER for all groups was at or above the expected TER for the intestinal barrier in clinically normal pigs, despite challenge inoculation with *Salmonella* organisms. In addition, the mean TER for *Salmonella*-challenged pigs fed 1.5 g of QBAs/1,000 kg of feed was 12 and 21 Ω/cm^2 greater than that of uninoculated control pigs (2 pigs that originated from the same source farm and had the same genetic background as the other pigs and were tested and found to be negative for *Salmonella* spp) and *Salmonella*-challenged pigs fed chlortetracycline, respectively. The QBAs appeared to have beneficial effects on intestinal barrier health, as indicated by an increase in TER. The potential mechanisms of action of QBAs are not currently known, but hypothesized mechanisms include modulation of gastrointestinal tract flora, enhanced intestinal protection, and repair of the intestinal epithelium. The specific mechanism of action by which QBAs improve intestinal health needs to be determined.

Although there are potential benefits of QBAs to food safety and public health through reduction in the shedding of *Salmonella* organisms prior to slaughter, their effects on the bacterial ecology and molecular epidemiology of bacteria commonly found within swine production units are unknown. The use of antimicrobials as growth promotants has been cited as a possible selective pressure responsible for the expansion of antimicrobial resistance within bacterial populations[5,27,28]; therefore, the potential influence of plant alkaloids (eg, QBAs) with antimicrobial qualities on the prevalence of antimicrobial resistance patterns of *Salmonella* spp recovered from naturally infected pigs should always be considered. Therefore, evaluation of pigs fed QBAs in a more representative production environment for the entire postweaning period is necessary before they can be confirmed as comparable or suitable alternatives to chlortetracycline for reducing fecal shedding of *Salmonella* organisms in swine in commercial production units.

a. Exede, Pfizer Animal Health, New York, NY.
b. Difco, Becton Dickinson, Sparks, Md.
c. Cathra replicator system, Oxoid Ltd, Basingstoke, Hampshire, England.
d. Excel, Microsoft Corp, Redmond, Wash.
e. Sangrovit, Phytobiotics GmBH, Eltville, Germany.
f. Aureomycin, Alpharma, Bridgewater, NJ.
g. Sensititre veterinary gram-negative MIC plate, TREK Diagnostics, Cleveland, Ohio.
h. MPN Calculator, build 23, Mike Curiale. Available at: members.ync.net/mcuriale/mpn/index.html. Accessed Feb 24, 2013.
i. Minitab, version 14, Minitab Inc, State College, Pa.
j. SAS, version 9.1, SAS Institute Inc, Cary, NC.

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