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Supplementation with Quaternary Benzo(c)phenanthridine Alkaloids Decreased Salivary Cortisol and *Salmonella* Shedding in Pigs After Transportation to the Slaughterhouse

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Abstract

The present study was aimed at evaluating the effect of herbal extracts supplementation, particularly quaternary-benzo(c)phenanthridine alkaloids (QBA), which have been previously demonstrated to have anti-inflammatory, antimicrobial, and immune-modulator effects. We investigated the role of QBA on stress response and *Salmonella* shedding in finishing pigs transported to the slaughterhouse. A total of 82 pigs were orally challenged with a *Salmonella* cocktail (day 0) containing *Salmonella* Meleagridis, Hartford, Bovismorbificans and Newport serovars and randomly assigned to three treatment groups after 2 wks (day [D] 14): T1, in-feed QBA; T2, in-feed and water-soluble QBA; CON, nonsupplemented). Pigs were transported to the slaughterhouse 2 weeks after intervention (D 28) and slaughtered after nearly 19 h (D 29). Saliva, fecal samples, and carcass swabs were collected from all pigs. Salivary cortisol, *Salmonella* shedding, and carcass contamination were measured. A high positive correlation (Spearman rank correlation coefficient range 0.82–0.93) between salivary cortisol and *Salmonella* shedding was found after transportation in all groups ($p < 0.05$). Only the CON group showed an increase in salivary cortisol after transportation (5.48 ng/mL; $p < 0.0001$) to concentrations that were higher than in T1 (2.73 ng/mL; $p = 0.0002$) and T2 (1.88 ng/mL; $p < 0.0001$). *Salmonella* prevalence and shedding decreased after transportation in pigs receiving the QBA intervention ($p < 0.05$), whereas the control group showed a significant increase in *Salmonella* shedding after transportation ($p = 0.04$). At D 28, pigs in T2 shed lower numbers of *Salmonella* as compared to T1 (1.3E + 02 CFU/mL versus 8E + 03 CFU/mL; $p = 0.002$). Additionally, carcass contamination by *Salmonella* was higher in the CON group than the treated groups ($p = 0.01$). The findings show QBA intervention was effective in reducing transportation stress of pigs, resulting in reduced *Salmonella* shedding and positively impacting animal welfare and pork safety.

Introduction

SALMONELLOSIS IS THE FOODBORNE disease with the highest hospitalization and death rates in the United States, costing approximately US$ 2.5 billion annually (Hoelzer *et al*., 2011). Furthermore, the consumption of contaminated pork has been associated with nearly 1% of all human salmonellosis (Guo *et al*., 2011). Pork can be contaminated with *Salmonella* at any point in the food chain; however, infected pigs entering the abattoir are considered the primary source of carcass contamination (Davies, 2011).

In pigs, salmonellosis is usually subclinical and infected pigs can shed *Salmonella* through the feces intermittently for long periods of time (Kranker *et al*., 2003). Stress due to transportation has been shown to increase *Salmonella* shedding even among those with subclinical infections at the on-farm stage, thus increasing the food safety risk (Larsen *et al*., 2003; Verbrugghe *et al*., 2011). Intervention strategies including the use of antibiotics have been extensively investigated to reduce the incidence of foodborne pathogens at the farm level (Doyle and Erickson, 2012; Looft *et al*., 2012). However, their extensive use has been associated with the development and spread of resistant bacteria (Oliver *et al*., 2009).

The use of herbal extracts, such as sanguinarine and chelerythrine, has been proposed as a good alternative to the use

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of in-feed antibiotics to enhance growth without promoting the development of antibiotic resistance (Yakhkeshi *et al*., 2011; Robbins *et al*., 2013). These compounds are quaternary benzo(c)phenanthridine alkaloids (QBA) and the biologically active components of several plants, including *Macleaya cordata,* which have been shown to have anti-inflammatory, antimicrobial, and immunomodulatory effects (Lenfeld *et al*., 1981; Colombo and Bosisio, 1996). Furthermore, QBA irreversibly inhibit the enzyme aromatic L-amino acid decarboxylase, which catalyzes decarboxylation of aromatic amino acids to their biogenic amines; thus, the availability of aromatic amino acids such as tryptophan is increased (Drsata *et al*., 1996). Tryptophan is an essential amino acid, and < 5% of total tryptophan is metabolized through the methoxyindoles pathway to synthesize the neurotransmitter serotonin, known to enhance stress adaptation. Therefore, the amount of serotonin synthesized and released directly depends on the availability of tryptophan (Oxenkrug, 2010; Shen *et al*., 2012a). These properties have encouraged the inclusion of QBA into swine and poultry diets to improve amino acid utilization and to promote growth, in countries where the use of QBA is approved (Vieira *et al*., 2008; Yakhkeshi *et al*., 2011). Additionally, our group previously reported that supplementation with QBA decreased *Salmonella* shedding and improved intestinal permeability in nursery pigs (Robbins *et al*., 2013). However, the effect of QBA supplementation on transportation-stress response in finishing pigs and its relevance to pork safety has not been investigated.

The objectives of the present study were to (1) evaluate the effect of QBA supplementation on salivary cortisol concentrations; (2) determine correlation between cortisol levels and *Salmonella* shedding; and (3) assess the effectiveness of QBA supplementation on *Salmonella* shedding and carcass contamination.

Methods

Animals and facilities

A total of 82 pigs (initial body weight: 47.9 ± 7.2 kg) were enrolled in a randomized controlled intervention study. Animals were blocked by litter, breed, and sex and randomly assigned to nine pens. Three consecutive pens were used for each treatment group and two empty pens and a solid barrier prevented the direct contact between different treatment groups. Pigs were housed in a partial slatted finishing barn at the Ohio Agricultural Research Development Center Western Agricultural Research Station, The Ohio State University. Feed and water were provided *ad libitum*. A biosecurity plan was implemented to minimize human exposure to *Salmonella* as well as to prevent its spread to other animals and facilities within the farm and the slaughterhouse. All procedures described below were approved by The Ohio State University Institutional Animal Care and Use Committee.

Oral Salmonella *challenge*

On day 0 (D 0), all pigs received 15 mL of a bacterial suspension *per os* containing a cocktail of *Salmonella* serovars isolated from fecal samples collected from the study pigs on the same farm 2 weeks prior to the beginning of the study by using standard procedures (Bager and Petersen, 1991). The resident *Salmonella* serovars included Bovismorbificans, Newport, Hartford, and Meleagridis. All isolates were pansusceptible to a panel of 12 antibiotics: ampicillin (10 μg), amoxicillin–clavulanic acid (30 μg), amikacin (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), sulfisoxazole (250 μg), and tetracycline (30 μg), according to the Clinical Laboratory Standards Institute (CLSI, 2012). The use of a cocktail containing the resident *Salmonella* serovars was intended to better simulate the real epidemiological scenario in a commercial farm. The identified *Salmonella* serovars are low pathogenic, causing mild and transient disease (Wood *et al*., 1991; Vigo *et al*., 2009; Ngoc *et al*., 2013; Jackson *et al*., 2013); therefore, all pigs received a booster challenge (15 mL) 9 days after (D 9) to ensure colonization. Both initial and booster challenges were prepared in Luria-Bertani broth and contained approximately 10^8 colony-forming units (CFU)/mL (0.5 in McFarland scale).

QBA treatments and administration

Fourteen days after the initial oral challenge (D 14), treatments and control groups were randomly assigned to the pens. Pigs in treatment 1 (T1; $n = 27$) received supplementation with 150 g QBA/ton of feed for 2 weeks (Shen *et al*., 2012a). Pigs in treatment 2 (T2; $n = 27$) received supplementation with 150 g QBA/ton of feed for 2 weeks and 100 g QBA/1000 L of drinking water during the last 3 days to augment the effect of in-feed QBA. No supplementation was given to pigs in the control group (CON; $n = 28$). QBA treatment consisted in a feed or water additive containing plant ingredients and natural extract of *Macleaya cordata*, with at least 1–1.5% sanguinarine (Sangrovit® and Sangrovit® WS; Phytobiotics GmbH, Eltville, Germany).

Transportation to the slaughterhouse

On day 28 (D 28), all pigs were transported to The Ohio State University Meat Lab slaughterhouse. In the trailer, a solid barrier prevented the direct contact between pigs from different treatment groups. After a transport period of about 45–50 min, all pigs were unloaded to the lairage (holding) pens and slaughtered after approximately 19 h (D 29). No direct (nose to nose) contact was allowed between pigs of different treatment groups during lairage, and the treatment groups were alternated during loading and slaughter to avoid potential confounders due to differences in loading and lairage time, which may affect the stress condition of the pigs.

Samples collection and processing

Saliva was collected from all pigs using a cotton swab (Salivette® Cortisol, Sarstedt AG & Co., Germany) on D 0, D 14, D 27 (pre-transport) and D28 (post-transport). To avoid confounding due to the circadian rhythmicity of cortisol, all samples were collected between 1 p.m. and 3 p.m., rotating the order of the treatment groups. Two weeks before the beginning of the study, all pigs were introduced and trained with the cotton swab to minimize stress due to the collection procedure, which may influence stress condition of the pigs, thus affecting salivary cortisol. Pigs were allowed to chew the cotton swab for approximately 60 s or until the cotton was

thoroughly saturated with saliva. The cotton swab was then placed into the inner part of the collector tube and transported to the laboratory on ice for further processing. Upon arrival, all samples were centrifuged at 1500 × *g* at 4°C for 10 min to separate saliva from the cotton swab (Shen *et al*., 2012b). Finally, saliva was stored at −80°C until further processing. A total of 310 saliva samples (T1, $n = 107$; T2, $n = 104$; CON, $n = 99$) were analyzed in duplicate for determination of salivary cortisol concentrations using a commercially available enzyme immunoassay kit (Salimetrics LLC, State College, PA). The intraclass coefficient of variation (CV) is calculated for each sample as: standard deviation/mean salivary cortisol (ng/mL)*100. Then, the average CV% is calculated for the 310 samples. The interassay CV was calculated using standard deviation/mean salivary cortisol (ng/mL)*100 from known low and high salivary cortisol controls included in each plate along with the standards and the unknown samples. Based on these methods, the intra- and interassay coefficients of variation were found to be 6% and 2.9%, respectively.

Fresh fecal matter was aseptically collected from the rectum of all pigs on D 0, D 3, D 14, D 21, D 27, and D 28 and individually placed into sterile bags (Nasco Whirl-Pak® Easy-To-Close; Fort Atkinson, WI) that were kept refrigerated until processing in the laboratory. A total of 384 fecal samples were collected (T1, $n = 129$; T2, $n = 130$; CON, $n = 125$) and the genomic DNA was extracted by using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions and the purified DNA was kept at 4°C until further analysis.

Carcass swabs were collected from all carcasses (internal and external surfaces) after evisceration and before final wash and chilling at slaughter (D 29) using sterile sponges (Nasco Whirl-Pak® Speci-Sponge®) premoistened with 10 mL of buffered peptone water. The sponges were kept on ice until processing in the laboratory. To extract the genomic DNA from the carcass swabs, a total of 72 sponges (T1, $n = 25$; T2, $n = 23$; CON, $n = 24$) were placed into individual sterile containers with 50 mL of a 0.02% Tween 20 (Sigma, St. Louis, MO) solution and agitated for 30 min in a shaker at 37°C. Subsequently, 15 mL of the liquid was poured into a sterile 15-mL Falcon tube (BD Falcon®; Franklin Lakes, NJ) and centrifuged at 1500 × *g* for 10 min to obtain the pellet (Guy *et al*., 2006). Finally, the DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen) following manufacturer's directions and the DNA was stored at 4°C until further analysis.

All fecal samples and carcass swabs were analyzed in triplicate for the quantification of *Salmonella* by performing a quantitative real-time polymerase chain reaction assay (qPCR) on an Mx3005P machine (Agilent Technologies, Santa Clara, CA). The set of primers selected were designed to amplify a 119-base pair fragment of the *invA* gene, as previously described (forward: 5'-TCGTCATTCCATTACCTACC-3' and reverse: 5'-AAACGTTGAAAAACTGAGGA-3'; Hoorfar *et al*., 2000). Thermal cycling conditions were as follows: 1 cycle at 95°C for 15 min (hot start), followed by 55 cycles of 95°C for 15 s (denature), 55°C for 15 s (annealing), 72°C for 30 s (extension) and 75°C for 15 s (additional data acquisition step), and 1 cycle at 72°C for 5 min (final extension). A standard curve was constructed by plotting known CFU/mL of *Salmonella* Enteritidis ATCC 13076 versus the threshold values (C_t), as previously described (Nam *et al*., 2005) and a linear regression analysis was performed with the MxPro® QPCR Software (Agilent Technologies) to quantify the amount of *Salmonella* (CFU/g or CFU/mL).

Statistical analysis

From 82 pigs originally enrolled in the study, 6 pigs (T1, $n = 1$; T2, $n = 2$; CON, $n = 3$) were removed because they died or received antibiotics before the end of the study period. For all continuous data, a test for normality of residuals was performed using the univariate procedure of SAS (SAS 9.4; Cary, NC) prior to statistical analysis. The means procedure of SAS (SAS 9.4) was used to calculate means and the standard error of the mean. Salivary cortisol was analyzed using the mixed procedure of SAS (SAS 9.4). Concentrations of salivary cortisol over time were analyzed as repeated measures. Pigs and pens were treated as a random effect. The final model included the effect of treatment, day of sample collection, their interaction, and sex. For *Salmonella* shedding, nonparametric tests were performed using the SAS software (SAS 9.4). The Kruskall–Wallis test was performed for the analysis of *Salmonella* shedding at D 0, D 3, D 14, D 21, D 27, D 28, and carcass contamination between treatment groups. Furthermore, the Wilcoxon rank sum (Mann–Whitney) test was performed to conduct pairwise comparisons between treatment groups. Pairwise comparisons were also performed within treatment groups with the Wilcoxon signed rank test. A logistic regression analysis was performed to test for differences in *Salmonella* prevalence between and within treatments by using the GLIMMIX procedure of SAS (SAS 9.4). Pigs and pens were treated as a random effect. The final model included the effect of treatment, day of sample collection, and their interaction. Due to convergence problems, the Fisher exact test was performed to test the significant differences in *Salmonella* prevalence between D 27 versus D 28 within treatments and between treatment groups at D 3 and D 28. *Salmonella* prevalence in carcass swabs was analyzed by performing a Fisher exact test. For all analyses, a p-value < 0.05 was considered significant and the Tukey–Kramer test was performed to adjust for multiple comparisons. The Spearman rank correlation coefficient (r_s) was calculated to determine the association between salivary cortisol and *Salmonella* shedding at D 28 and the size of the correlation coefficient was interpreted as described previously (Mukaka, 2012).

Results

The Spearman rank correlation coefficient revealed a very high to a high positive association between salivary cortisol and *Salmonella* shedding after transportation in all groups, with the highest r_s in the CON group (Fig. 1; $r_s = 0.93$, $p < 0.001$), followed by T1 ($r_s = 0.85$, $p = 0.0002$) and T2 ($r_s = 0.82$, $p = 0.0006$).

Salivary cortisol

Overall, mean concentrations of salivary cortisol decreased significantly in all groups from D 0 to D 27 (Fig. 2; $p < 0.0001$). After transportation to the slaughterhouse (D 28) only the CON group showed a significant increase in salivary cortisol as compared to D 27 (1.87 ng/mL to 5.48 ng/mL, $p < 0.0001$). Additionally, the mean salivary cortisol on D 28

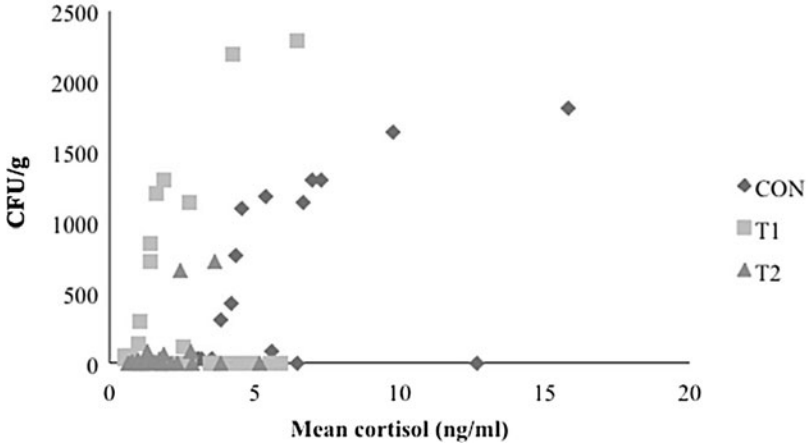

FIG. 1. Spearman correlation between *Salmonella* shedding (colony-forming units [CFU]/g feces) and salivary cortisol concentrations (ng cortisol/mL saliva) after transportation to the slaughterhouse among treatments and control groups. CON (control with basal diet); T1 (in-feed quarternary benzo(c)phenanthridine alkaloids [QBA]) and T2 (in-feed + water-soluble QBA).

was significantly higher in the CON group than in T1 (5.48 ng/mL versus 2.73 ng/mL, $p = 0.0002$) and T2 (5.48 ng/mL versus 1.88 ng/mL, $p < 0.0001$).

Salmonella *fecal shedding*

Salmonella prevalence ranged from 71.4% to 100% and it was not different between treatment groups at D 0, D 3, D 14, D 21, and D 27 ($p > 0.05$), indicating that the challenge worked effectively and shedding was persistent throughout the study period (data not shown). On D 28, the prevalence of *Salmonella* was significantly higher in pigs in the CON group (100%) as compared to T1 (73.7%, $p = 0.02$) and T2 (71.4%, $p = 0.02$). Additionally, the proportion of *Salmonella*-positive pigs in T2 decreased significantly after transportation as compared to D 27 (95.5% to 71.4%, $p = 0.05$).

Salmonella shedding was significantly different between groups at D 27 (Fig. 3; $p = 0.0002$) and D 28 ($p = 0.01$). At D 27 (before transportation stress), pigs in the CON group shed lower amounts of *Salmonella* as compared to pigs receiving either in-feed QBA ($p < 0.0001$) or in-feed and water soluble QBA ($p = 0.001$). At D 28 (after transportation stress event), pigs in T2 shed significantly lower numbers of *Salmonella* as compared to T1 (1.3E + 02 CFU/g versus 8E + 03 CFU/g, $p = 0.002$), implying a better response when QBA was added to the drinking water, perhaps due to a higher bioavailability (del Castillo *et al*., 1998). However, the differences were not statistically significant between T2 and the CON group (1.3E + 02 CFU/g versus 5.9E + 02 CFU/g, $p = 0.08$). In addition, pigs in the CON group showed a significant increase in *Salmonella* shedding after transportation (6E + 01 CFU/g to 5.9E + 02 CFU/g, $p = 0.04$). Conversely, pigs in T2 exhibited a significant decrease in *Salmonella* shedding after transportation as compared to pretransport levels (3.8E + 02 CFU/g to 1.3E + 02 CFU/g, $p = 0.03$).

Carcass contamination with Salmonella

Salmonella was detected in 37.5% (27/72) of all carcasses. *Salmonella* prevalence was not significantly different between any of the treatment groups: QBA (T1 and T2 combined) versus CON group ($p = 0.32$), T1 versus CON ($p = 0.07$), T2 versus CON ($p = 1$) or T1 versus T2 ($p = 0.07$). However, the quantity of *Salmonella* contaminating the carcasses was significantly different between treatment groups (Fig. 4; $p = 0.03$). The amount of *Salmonella* contaminating the carcasses in the CON group was significantly higher as compared to both T1 and T2 (3.7E + 01 CFU/mL versus 9E + 00 CFU/mL and 1E + 01 CFU/mL respectively, $p = 0.01$).

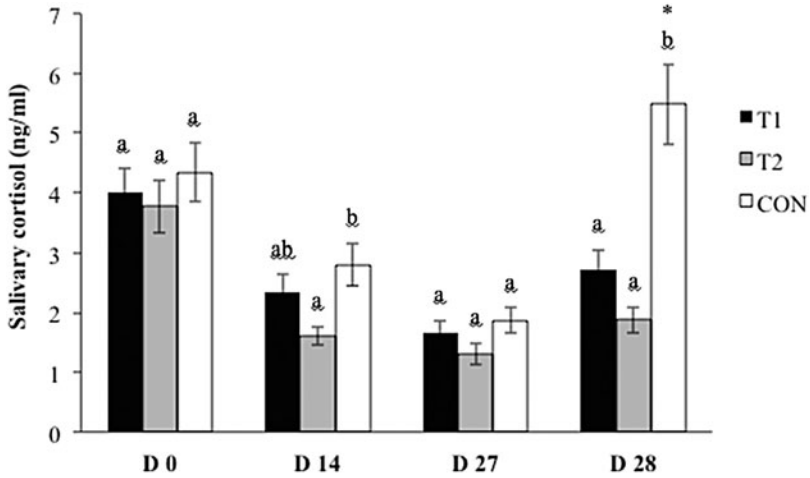

FIG. 2. Comparison of salivary cortisol concentrations (ng cortisol/mL saliva) among treatments and control groups. CON (control with basal diet); T1 (in-feed quarternary benzo(c)phenanthridine alkaloids [QBA]) and T2 (in-feed + water soluble QBA). D 27 (Day 27, pre-transportation) and D 28 (Day 28, after transportation to slaughter). [a,b]Different letters denote significance between treatment groups at $p < 0.05$. *Denotes significance within a treatment between D 27 and D 28 at $p < 0.05$.

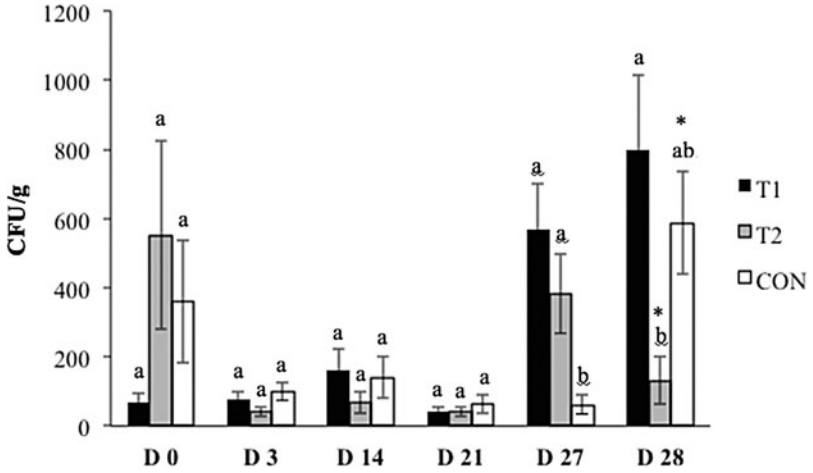

FIG. 3. Comparison of *Salmonella* shedding levels (colony-forming units [CFU]/g feces) among treatments and control groups. CON (control with basal diet); T1 (in-feed quarternary benzo(c)phenanthridine alkaloids [QBA]) and T2 (in-feed + water-soluble QBA). D 27 (Day 27, pretransportation) and D 28 (Day 28, after transportation to slaughter). [a,b]Different letters denote significance between treatment groups at $p < 0.05$. *Denotes significance within a treatment between D 27 and D 28 at $p < 0.05$.

Discussion

The primary findings of this study are the following: (1) transportation is a stressful event for pigs, resulting in increased salivary cortisol and *Salmonella* shedding; and (2) adding QBA to the feed and the drinking water was effective in regulating transportation stress response and reducing *Salmonella* prevalence and the quantity of *Salmonella* shed through their feces.

Transportation to the slaughterhouse is one of the most stressful events for pigs, which can negatively affect the normal functioning of the gastrointestinal tract (Webster Marketon and Glaser, 2008; Rostagno, 2009). Moreover, stress has been associated with increased *Salmonella* shedding and a higher food safety risk (Rostagno, 2009). Our results are in accordance with previous reports and showed that only pigs in the CON exhibited an increase in salivary cortisol after transportation, indicating that transportation is truly a stressor for finishing pigs. Additionally, only non-supplemented pigs showed a significant increase in *Salmonella* shedding after transportation. Furthermore, our results, in accordance with other studies (Hurd *et al*., 2002; Verbrugghe *et al*., 2011), showed a high positive correlation between salivary cortisol and *Salmonella* shedding after transportation, suggesting that regulating stress may be a good strategy to decrease *Salmonella* shedding after transportation to the slaughterhouse.

In addition to other physiological effects, QBA have been shown to decrease *Salmonella* shedding in nursery pigs (Robbins *et al*., 2013). In agreement with Robbins *et al*. (2013), the current results showed a significant decrease in the proportion of *Salmonella*-positive pigs as well as in the amount of *Salmonella* shed through the feces after transportation to the slaughterhouse when QBA were included in the feed and the drinking water. Additionally, pigs in the T2 tended to shed lower amounts of *Salmonella* as compared to the CON group ($p = 0.08$) after transportation. Therefore, the results showed that in-feed and water QBA supplementation was effective in reducing *Salmonella* shedding after transportation to the slaughterhouse.

In the present study, about 38% of all carcasses were contaminated with *Salmonella* and similar results were previously reported (Botteldoorn *et al*., 2003; Arguello *et al*., 2013). Carcass contamination by *Salmonella* was significantly higher in the CON group as compared to T1 and T2. Moreover, pigs in T2 tended to shed fewer *Salmonella* after transportation as compared to the CON group, suggesting that pigs in T2 entering the slaughter line were not as highly infected as pigs of the CON group, thus reducing the risk of carcass contamination with highly contaminated feces. In the present study, the analysis of salivary cortisol concentrations showed a significantly lower stress response after transportation in pigs of T1 as compared to the CON group. However, the lower stress response in T1 did not result in a decrease in

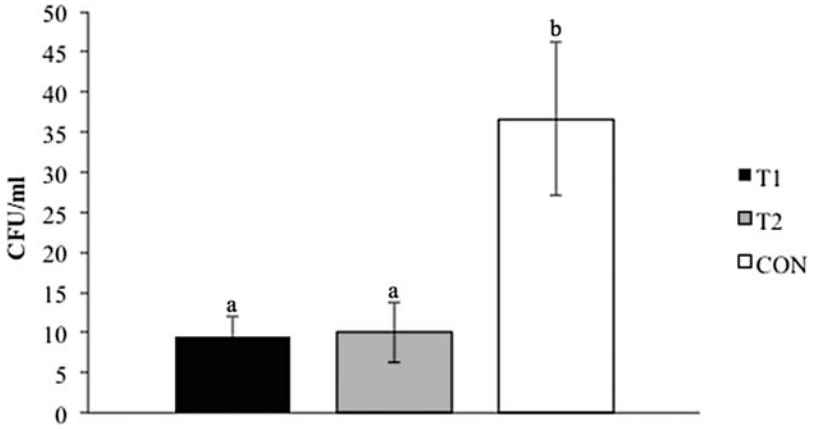

FIG. 4. Overall comparison of carcass contamination with *Salmonella* (colony-forming units [CFU]/mL) among treatments and control groups. CON (control with basal diet); T1 (in-feed quarternary benzo(c)phenanthridine alkaloids [QBA]) and T2 (in-feed + water-soluble QBA). [a,b]Different letters denote significance between treatment groups at $p < 0.05$.

the shedding of *Salmonella* after transportation to the slaughterhouse. The significantly lower contamination of the carcasses in T1 suggested a better adaptation to stress during lairage that might have decrease the shedding of *Salmonella* in pigs entering the abattoir and thus, the risk of carcass contamination. More research is needed to elucidate the mechanism by which QBA supplementation ameliorates stress adaptability and *Salmonella* shedding during lairage.

In conclusion, results from this study indicated that adding QBA to the feed and the drinking water of finishing pigs was effective in reducing both the proportion of *Salmonella*-positive pigs and the amount of *Salmonella* shed after transportation to the slaughterhouse that can potentially contaminate the carcasses. Additionally, the results suggested that sanguinarine and chelerythrine supplementation was successful in reducing carcass contamination with *Salmonella*. Furthermore, QBA supplementation was effective in regulating stress response due to transportation, which might have decreased the negative impact of stress on the gastrointestinal tract, decreasing the shedding of *Salmonella*. Further research is needed to determine underlying mechanisms for reducing *Salmonella* shedding and reducing salivary cortisol. Additionally, more research is necessary to evaluate the effect of QBA supplementation on antibiotic resistance in *Salmonella*.

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Disclosure Statement

No competing financial interests exist.

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