Journal of Food Protection, Vol. 75, No. 4, 2012, Pages 758–761
doi:10.4315/0362-028X.JFP-11-390

Research Note

Effect of Thymol or Diphenyliodonium Chloride on Performance, Gut Fermentation Characteristics, and *Campylobacter* Colonization in Growing Swine†‡

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MS 11-390: Received 22 August 2011/Accepted 14 December 2011

ABSTRACT

Food producing animals can be reservoirs of *Campylobacter,* a leading bacterial cause of human foodborne illness. *Campylobacter* spp. utilize amino acids as major carbon and energy substrates, a process that can be inhibited by thymol and diphenyliodonium chloride (DIC). To determine the effect of these potential additives on feed intake, live weight gain, and gut *Campylobacter* levels, growing pigs were fed standard grower diets supplemented with or without 0.0067 or 0.0201% thymol or 0.00014 or 0.00042% DIC in a replicated study design. Diets were offered twice daily for 7 days, during which time daily feed intake (mean ± SEM, 2.39 ± 0.06 kg day^{-1}) and daily gain (0.62 ± 0.04 kg day^{-1}) were unaffected ($P > 0.05$) by treatment. Pigs treated with DIC but not thymol tended to have lower rectal *Campylobacter* levels ($P = 0.07$) (5.2 versus 4.2 and 4.4 log CFU g^{-1} rectal contents for controls and 0.00014% DIC and 0.00042% DIC, respectively; SEM = 0.26). However, DIC or thymol treatments did not affect ($P > 0.05$) ileal or cecal *Campylobacter* (1.6 ± 0.17 and 4.5 ± 0.26 log CFU g^{-1}, respectively), cecal total culturable anaerobes (9.8 ± 0.10 log CFU g^{-1}), or accumulations of major fermentation end products within collected gut contents. These results suggest that thymol and DIC were appreciably absorbed, degraded, or otherwise made unavailable in the proximal alimentary tract and that encapsulation technologies will likely be needed to deliver effective concentrations of these compounds to the lower gut to achieve in vivo reductions of *Campylobacter*.

Campylobacter infection is a leading bacterial cause of human foodborne disease, responsible for 13 reported cases per every 100,000 persons per year in the U.S. population *(4)* at an annual cost of more than $1.2 billion *(7)*. Whereas most human *Campylobacter* infections are caused by *Campylobacter jejuni,* an estimated 26,000 infections are caused annually by *Campylobacter coli,* which is the predominant *Campylobacter* species found in swine *(10, 20)*. Both *Campylobacter* species are recognized as important reservoirs of antimicrobial resistance genes that can be transmitted to other pathogenic or commensal bacteria *(21, 22)*. Consequently, an important step toward lowering numbers of foodborne illnesses would be to reduce the colonization and carriage of *Campylobacter* in food animals before they arrive for processing.

Campylobacter lacks 6-phosphofructokinase *(26)* and thus is limited in its ability to obtain carbon or energy from sugars or carbohydrates. *Campylobacter* may conserve energy via respiration using a variety of electron acceptors *(13),* but because concentrations of anaerobic electron acceptors are typically low in gut environments *Campylobacter* growth also may be limited. Recent evidence has revealed strain-specific uptake and utilization of L-fucose by a putative fucose-permease expressed by *C. jejuni*; however, this ability is not necessarily required to promote colonization of the chick ceca *(14)*. Conversely, most *Campylobacter* strains utilize amino acids such as alanine, aspartate, glutamate, glutamine, methionine, and serine as carbon and energy substrates *(16, 27),* and the ability to metabolize these amino acids enables colonization of the gut of animals. For instance, a *C. jejuni* mutant lacking L-serine dehydratase activity and thus deficient in L-serine catabolism was unable to colonize the avian gut *(25)*. The ability of a virulent *C. jejuni* strain to catabolize aspartate and glutamate or glutamate precursors reportedly promoted this strain's ability to colonize the host *(8, 9)*.

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† Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

‡ Results of this study were presented in preliminary form at the 8th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork in Quebec City, Canada, 30 September to 2 October 2009.

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Results of a previous study suggested that inhibition of amino acid catabolism using the purported deaminase inhibitors diphenyliodonium chloride (DIC) and thymol *(2)* may explain the observed reductions (>5 log) in survivability of *C. jejuni* and *C. coli* during incubation with swine fecal microbes in vitro *(1)*. Although these study results confirmed that DIC inhibited enzymatic catabolism of amino acids, as evidenced by significant reductions in specific ammonia-producing activity by crude *C. jejuni* and *C. coli* enzyme preparations, the case was less clear with thymol. This inhibitor reduced the specific ammonia-producing activity by enzyme preparations of *C. coli* but not of *C. jejuni (1)*. Consequently, we could not clearly determine whether the bactericidal effect of thymol against *Campylobacter* was due to an inhibition of deaminase, as suggested by Broderick and Balthrop *(2),* or to the disruption of the bacterial cell wall as more generally thought by others *(3, 17)*. Regardless of the mode of action, results from our earlier in vitro studies clearly indicated that DIC and thymol are bactericidal against *C. coli* and *C. jejuni* and thus may be effective for reducing the carriage of important foodborne pathogens in animals. However, no one has determined whether pigs will find these inhibitors palatable or whether concentrations that reduce *Campylobacter* in vitro will likewise be effective in vivo. The primary objective of the present study was to determine the effect of thymol or DIC diet supplements on feed intake, live weight gain, and gut *Campylobacter* levels in a weaned pig model.

MATERIALS AND METHODS

In two separate trials, weaned pigs approximately 6 weeks of age were purchased from the Texas A&M University Animal Science Department and transported to the Southern Plains Agricultural Research Center's swine grow-out facility. The experimental trials were conducted in accordance with procedures approved by the Center's Animal Care and Use Committee. The pigs were randomly allocated to individual pens (two pigs per pen) and acclimated to a standard commercial grower diet (Producers Cooperative Association, Bryan, TX) for 3 weeks, after which pigs were fed diets supplemented with 0×, 1×, or 3× DIC or thymol, each purchased from Sigma-Aldrich (St. Louis, MO), where 1× is equivalent to 0.00014% DIC and 0.0067% thymol (each on a wt/wt basis).

Treatments were administered by spraying each meal with 3 ml of concentrated stock solutions of DIC (2.13 or 6.39 mM prepared in water) or thymol (212.92 or 638.76 mM prepared in ethanol). Meals were offered at approximately 07:30 and 16:30 each day; consequently, each pen was considered an experimental unit ($n = 4$ per treatment in each trial). Amounts of feed offered and refused were measured daily during a 7-day period for the first replicate, but because of scheduling conflicts these measurements were taken on only days 3, 5, 6, and 7 in the second replicate. Samples of feed offered and refused were collected, homogenized, and dried at 100°C until a constant weight was achieved, and the resultant dry matter percentages were used to adjust intakes to a dry matter basis.

On day 8 after initiation of treatment, all pigs were euthanized via intravenous sodium pentobarbital injection and necropsied for collection of ileal, cecal, and rectal contents. Contents were serially diluted into sodium phosphate–buffered saline (pH 6.5) and plated onto Campy Cefex agar prepared and used as described by Stern et al. *(19)* for enumeration of *Campylobacter* as routinely done in our laboratory *(12)*. Colonies were counted after 48 h of microaerobic incubation (N_2:CO_2:O_2 = 85:10:5) at 42°C. Cecal contents also were diluted into anaerobic buffer that was prepared under 100% N_2 and plated onto *Brucella* blood agar (Anaerobe Systems, Morgan Hill, CA) for enumeration of total culturable anaerobic bacteria. Colonies were counted after 4 days of incubation at 39°C in a Bactron IV Anaerobic/Environmental Chamber (Sheldon Manufacturing, Cornelius, OR) under an N_2:CO_2:H_2 atmosphere of 90:5:5. Ammonia concentrations in gut contents were measured with a colorimetric procedure *(6),* and volatile fatty acid concentrations were measured by gas chromatography *(18)*.

A general analysis of variance was used to test for potential effects of treatment on dry matter intake, average daily gain, and gut concentrations of volatile fatty acids, ammonia, and log-transformed bacterial counts. The model statement included terms for treatment, replicate, pen, and pig, and a two-sided Dunnett multiple comparison procedure was used to compare treatment means with those of controls. All analyses were performed using STATISTIX9 Analytical Software (Tallahassee, FL).

RESULTS AND DISCUSSION

Mean daily dry matter intake (2.33, 2.30, 2.42, 2.36, and 2.42 kg day^{-1}; SEM = 0.06 kg day^{-1}) did not differ ($P > 0.05$) among control, 1× and 3× thymol, and 1× and 3× DIC treatments, respectively. Similarly, mean daily gain calculated for the 1-week feeding trials (0.64, 0.62, 0.61, 0.62, and 0.59 kg day^{-1}; SEM = 0.04 kg day^{-1}) did not differ ($P > 0.05$) between treatments. This result was expected because 1 week would likely be too short a time to adequately measure the effects of these compounds on daily gain. Accordingly, we concluded that the amounts of thymol and DIC administered in this study did not negatively affect feed intake. Jugl-Chizzola et al. *(11)* reported decreased feed intake by weaned pigs fed diets containing 1% (wt/wt) thyme (containing the equivalent 0.02% thymol) but not by pigs fed 0.1% thyme (the equivalent of 0.002% thymol). Trevisi et al. *(23)* reported a similar decreased feed intake. These investigators also did not find adverse effects on weight gain by weaned pigs fed diets containing 1% (wt/wt) thymol compared with pigs fed unsupplemented control diets.

Campylobacter levels recovered from ileal and cecal contents of thymol- or DIC-treated pigs at necropsy did not differ from those recovered from pigs fed the unsupplemented control diet (Table 1). Similarly, levels of total culturable anaerobes recovered from cecal contents were unaffected by 1× or 3× thymol (9.7 and 9.7 log CFU g^{-1}, respectively) or DIC treatment (9.8 and 9.7 log CFU g^{-1}, respectively) compared with controls (9.9 log CFU g^{-1}) (SEM = 0.10 log CFU g^{-1}). Although the duration of the present study may have been too short to allow sufficient time for the treatments to exert their effects, it is more likely that the inhibitors were appreciably absorbed, degraded, or irreversibly bound to digesta within the proximal alimentary tract. Each treated pig ingested thymol at an average of 77 and 243 mg day^{-1} for the 1× and 3× treatments, respectively, and DIC at 1.6 and 5.1 mg day^{-1} for the 1× and 3× treatments, respectively. If 100% of each

TABLE 1. Campylobacter *levels in gut contents from weaned pigs fed diets supplemented with or without thymol or diphenyliodonium chloride (DIC)*

Sample	Mean *Campylobacter* (log CFU g^{-1})					SEM (log CFU g^{-1})	*P* value
	Control	1× thymol	3× thymol	1× DIC	3× DIC		
Ileal	1.76	1.66	1.56	1.41	1.75	0.17	0.5316
Cecal	4.82	4.53	4.15	4.24	4.69	0.26	0.3285
Rectal	5.22	4.48	4.69	4.23[a]	4.38[b]	0.26	0.0707

[a] Mean differs from that of controls at $P < 0.05$.
[b] Mean differs from that of controls at $P < 0.10$.

consumed dose had passed to the lower gut, then luminal concentrations would have been 0.8 and 2.5 mM thymol and 0.008 and 0.025 mM DIC, respectively (based on an estimated lower gut volume of 640 ml). These concentrations would exceed or be very close to concentrations previously determined to be efficacious in vitro *(1)*. Michiels et al. *(15)* also reported the near complete absorption of thymol within the pig stomach and small intestine when administered as a single dose at 13.2 mg kg^{-1} body weight. Although Michiels et al. *(15)* observed no degradation of thymol during test incubations simulating stomach and jejunal contents, approximately 20 to 30% of the added thymol was degraded during in vitro incubations with simulated cecal contents. Little is known regarding the potential absorption or degradation of DIC in the pig gastrointestinal tract.

Rectal *Campylobacter* levels from thymol-treated pigs did not differ from those of controls. However, *Campylobacter* levels tended ($P = 0.07$) to be lower in rectal contents collected from pigs fed diets supplemented with 1× or 3× DIC than in those from pigs fed the control diet, but it is unclear whether this difference can be directly attributed to luminal concentrations of DIC or to potential secondary effects of the inhibitor. Our finding that concentrations of ammonia and the volatile fatty acids acetate, propionate, and butyrate in ileal, cecal, and rectal contents were unaffected by thymol or DIC treatment ($P > 0.05$) again suggests that the amounts of these inhibitors reaching these gastrointestinal sites were not high enough to affect production of these fermentative end products (Table 2).

Thymol and DIC have been investigated as feed additives that can be used to reduce the energetically wasteful process of ruminal protein degradation in cattle without producing adverse effects on animal health *(2, 5)*. Varel and Miller *(24)* recognized the deaminase inhibiting potential of DIC for controlling gaseous emissions from livestock waste, and in vitro studies revealed that inhibition of amino acid metabolism by these chemicals may be a metabolic target for the control of foodborne *Campylobacter (1)*. Results from the present study indicated that neither thymol nor DIC negatively affected dry matter intake or live weight gain when fed for 7 days in diets supplemented with less than or equal to 0.0201% thymol or 0.00042% DIC (wt/wt). However, these treatments were not effective for reducing *Campylobacter* in the lower gut, most likely because these inhibitory compounds were absorbed or degraded in the stomach or small intestine. Although these

TABLE 2. *Fermentation end product concentrations in gut contents from weaned pigs fed diets supplemented with or without thymol or diphenyliodonium chloride (DIC)*

End product	Concn (μmol g^{-1})					SEM (μmol g^{-1})	*P* value
	Control	1× thymol	3× thymol	1× DIC	3× DIC		
Ileal							
Acetate	1.76	1.63	1.53	1.79	1.79	0.24	0.9142
Propionate	0.49	0.20	0.18	0.27	0.34	0.09	0.1137
Butyrate	0.18	0.11	0.10	0.15	0.14	0.04	0.5247
Ammonia	0.001	0.001	ND[a]	0.004	ND	0.01	0.4922
Cecal							
Acetate	9.72	8.51	9.57	11.04	9.69	0.82	0.3204
Propionate	5.98	4.48	5.10	5.77	5.41	0.58	0.3908
Butyrate	2.47	2.13	3.19	2.58	2.29	0.33	0.2052
Ammonia	0.005	0.021	0.005	0.012	0.018	0.01	0.8661
Rectal							
Acetate	6.90	6.41	5.78	6.35	6.76	0.61	0.7355
Propionate	2.90	2.69	2.22	2.67	2.86	0.32	0.6021
Butyrate	1.60	1.43	1.29	1.33	1.57	0.20	0.7608
Ammonia	0.202	0.185	0.120	0.169	0.172	0.05	0.8038

[a] ND, not determined.

compounds were effective for killing *Campylobacter* in vitro *(1),* in the present in vivo study they did not appear to be effective for reducing *Campylobacter* in the swine intestinal tract. These results indicate that encapsulation or other protective technologies will likely be needed to deliver effective concentrations of these compounds to the lower gut to enhance their efficacy against *Campylobacter* in growing swine.

ACKNOWLEDGMENTS

This work was funded in part with a grant from the National Pork Board (NPB 08-020). The authors thank Deb Lebo and Jackie Kotzur for their expert technical assistance.

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