

Research Note: Evaluation of deoxycholic acid for antihistomonal activity

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ABSTRACT Deoxycholic acid (DCA) is a naturally occurring secondary bile acid that originates from intestinal bacterial metabolic conversion of cholate, a primary bile acid. Deoxycholic acid was shown to have antihistomonal properties in vitro, leading to our hypothesis that DCA inclusion within the feed might prevent histomoniasis. Selected dietary concentrations of DCA were evaluated for effects on body weight gain (BWG), lesions, and mortality of turkeys challenged with wild-type *Histomonas meleagridis* (WTH). Treatments consisted of non-challenged control (NC; basal diet), 0.25% DCA diet + challenge, 0.5% DCA diet + challenge, 1% DCA diet + challenge, and a positive-challenged control (PC; basal diet). All groups were fed a basal starter diet until day 7, at which time DCA diets were administered to the respective groups. On day 14, 2×10^5 WTH cells/turkey were intracloacally administered. *H. meleagridis*-related lesions were evaluated on day 13 post-challenge. Pre-challenge day 0 to 14 BWG was higher ($P \leq 0.05$) in the

0.25% DCA group than in the 1% DCA group. There were no significant differences in pre-challenge day 0 to 14 BWG between any of the other groups. No significant differences in mortalities from histomoniasis occurred in the DCA groups as compared to the PC group. No *H. meleagridis* lesions or mortalities were observed at any time in the NC group. Presence of *H. meleagridis*-related liver lesions was higher ($P \leq 0.05$) in the 0.5% DCA group as compared to the PC group. Using the same controls and experimental timeline, an additional group was included to evaluate a biliogenic diet formulated with 20% whole egg powder to encourage endogenous bile acid production. The biliogenic diet had no statistical impact on pre-challenge day 0 to 14 BWG, but did not reduce *H. meleagridis*-related mortalities or lesions after the challenge. Taken together, these data suggest that DCA inclusion within the feed at these concentrations and under these experimental conditions does not prevent histomoniasis.

Key words: blackhead, deoxycholic acid, histomoniasis, *Histomonas meleagridis*, turkey

2020 Poultry Science 99:3481–3486

<https://doi.org/10.1016/j.psj.2020.03.049>

INTRODUCTION

Histomoniasis (synonyms: blackhead or infectious enterohepatitis) is an important disease primarily affecting turkeys in addition to other gallinaceous birds (van der Heijden et al., 2005; Hess et al., 2015). Caused by the protozoan parasite *Histomonas meleagridis*, mortality can approach 80 to 100% of the flock with significant economic damage incurred (Callait et al., 2002; McDougald, 2005; Hess and

McDougald, 2013). Nitroimidazoles were previously an effective treatment for histomoniasis; however, regulatory action resulted in the removal of effective prophylactic and therapeutic compounds without any alternatives introduced for disease treatment (Joyner, 1963; Hess and McDougald, 2013; Liebhart et al., 2013). Nitarsone, the last remaining Food and Drug Administration approved drug for the treatment of histomoniasis, was removed from the market in 2015 (Regmi et al., 2016). Due to the removal of these compounds, the development of an efficacious chemoprophylactic is needed.

Bile acids function as biological detergents, aiding in the production of antimicrobial peptides and contributing to host defense against pathogens (Winston and Theriot, 2016). Deoxycholic acid (DCA) is a naturally occurring secondary bile acid produced by intestinal bacterial metabolic conversion of cholate (van Eldere et al.,

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Received November 21, 2019.

Accepted March 21, 2020.

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1996; Ridlon et al., 2006). Increased secondary bile acids have been associated with the etiologies of cholesterol gallstone disease and colon carcinogenesis (Winston and Theriot, 2016). Considering these impacts on eukaryotic cells, we considered the potential toxicity of DCA to *H. meleagridis*. When administered at a dietary concentration of 0.0015%, DCA has been shown to reduce the severity of *Eimeria maxima* and *Clostridium perfringens* infections in poultry along with an associated reduction of damaged intestinal villi (Wang et al., 2019). A study with mice demonstrated that anaerobic bacteria-derived DCA protected against colitis that was induced by *Campylobacter jejuni* (Sun et al., 2018). Similarly, dietary supplementation with DCA reduced experimental *C. jejuni* colonization in chickens and affected composition of the microbiota (Alrubaye et al., 2019). This adoptive transfer of DCA-modulated microbiota suggests that the anti-*C. jejuni* effect was due to DCA-induced changes to composition and functionality of the microbiota.

Considering the reduced severity of protozoal and bacterial impacts following DCA treatment, we hypothesized that DCA might confer antihistomonal properties. The primary purpose of the present study was to evaluate DCA as a chemoprophylaxis candidate against histomoniasis in turkeys. Additionally, dietary composition has been shown to influence endogenous production of bile acids within the host (LeBlanc et al., 1998; Yang et al., 2012). Based upon these observations (LeBlanc et al., 1998; Yang et al., 2012), a putative biliogenic diet was formulated to encourage endogenous bile acid formation with the hypothesis that severity of histomoniasis would be reduced.

MATERIALS AND METHODS

H. meleagridis Isolate and Culture

A virulent, wild-type strain of *H. meleagridis* (WTH) was isolated from a field outbreak of histomoniasis that occurred in a flock of chickens (layer pullets) in the southern United States. Cells were cultured based on previously described methods (van der Heijden et al., 2005; van der Heijden and Landman, 2007; Hauck et al., 2010). Modified Dwyer's medium (MDM) was used for *H. meleagridis* cultivation and comprised Medium 199 (Catalog #12350-039; Gibco, Life Technologies Corporation, Waltham, MA) supplemented with 10% heat-inactivated horse serum (Catalog #26050-088; Gibco) and 1.6 mg/mL white rice flour (Arrowhead Mills, Boulder, CO). This *H. meleagridis* isolate contained an undefined polyxenic bacterial population. Culture flasks (Catalog #10062-874; VWR International LLC, Radnor, PA) were incubated anaerobically at 40°C for 48 to 72 h before 1 mL was subcultured into 12.5 mL of fresh, supplemented MDM. Growth was confirmed by observation with an inverted microscope and enumeration with a hemocytometer.

In vitro Assessment of DCA

Three *in vitro* assays were completed to evaluate selected concentrations of high purity DCA sodium salt (Catalog #97062-026; VWR International LLC) on inhibition of *H. meleagridis* proliferation. For these *in vitro* assays, WTH cells were propagated in MDM and selected from flasks containing high initial populations. Subsequently, WTH cells were added at a ratio of 100 μ L cells: 50 μ L DCA treatment into a 96-well, sterile microtiter plate. Each treatment was performed in pentaplicate. Following incubation under anaerobic condition at 40°C, viable cells were enumerated by trypan blue (0.4%) vital dye exclusion (Catalog #15250-061; Gibco) using a hemocytometer, and cell counts were expressed as viable cells/mL. Sufficient fields of vision were evaluated in order to reach a cell count ≥ 100 cells, as applicable.

In assay 1, a concentration of 2.01×10^6 WTH cells/mL was added according to the method above. Treatments included sterile PBS as a negative control or final concentrations of 0.4, 2, or 4 mM DCA. The plate was incubated for 7 to 8.5 h before viable cells were enumerated as described above. In assay 2, a concentration of 6.88×10^5 WTH cells/mL was used and treatments included either PBS control or final concentrations of 0.5, 1, 2, or 4 mM DCA. The plate was incubated for 6 to 8 h before viable cells were enumerated. In assay 3, a concentration of 6.35×10^5 cells/mL was added and treatments included either PBS or final concentrations of 0.5, 1, or 2 mM DCA. The plate was incubated and viable cells were enumerated at 2 time periods of 4 to 6 and 27 to 29 h after incubation.

Animal Source and Diet

A total of 140 day-of-hatch female turkey poults were obtained from a local commercial hatchery. Poults were neck-tagged individually and randomly allocated to floor pens at the University of Arkansas Poultry Health Laboratory. All animal handling procedures were in compliance with regulations of the Institutional Animal Care and Use Committee (IACUC protocol #18113) of the University of Arkansas. A corn soy-based starter feed that met or exceeded nutrient requirements for poultry (NRC, 1994) and water were provided *ad libitum*. Early poult mortalities unrelated to histomoniasis were recorded and altered group numbers were reported in the experiment.

Deoxycholic Acid Diet On day 7, DCA was included in the diet at selected concentrations of either 0.25, 0.5, or 1%. Treatments consisted of non-challenged control (NC; basal diet; n = 59), 0.25% DCA diet + challenge (n = 20), 0.5% DCA diet + challenge (n = 20), 1% DCA diet + challenge (n = 20), and positive-challenged control (PC; basal diet; n = 20). Turkeys remained on treatment diets for the remainder of the experiment.

Biliogenic Diet Using the same NC and PC groups as above, a separate group (n = 20) receiving a putative biliogenic diet consisting of 20% whole egg powder (Heartland Supply Co., Fayetteville, AR) inclusion within a

basal turkey starter was evaluated (Table 1), based upon previously published research (LeBlanc et al., 1998; Yang et al., 2012). The objective purpose of this treatment was to physiologically upregulate natural bile acid synthesis to potentially increase endogenous DCA production within the turkey. This group was subjected to the same experimental timeline and evaluation methods as the DCA treatment groups (Figure 1).

***H. meleagridis* Challenge**

Approximately 6 D before cells were needed for challenge or vaccination, the desired *H. meleagridis* aliquot was thawed from liquid nitrogen and cultured into fresh supplemented MDM according to the method described above. Following incubation, viable histomonads/mL were enumerated by trypan blue vital dye exclusion with a hemocytometer. Within each experiment, MDM was used as the diluent to prepare the proper dosage concentration. To initiate disease challenge, poults were intracloacally challenged with a gavage needle at a total dose of 2×10^5 WTH cells/turkey on day 14 of age. The inoculation was divided into 2 administrations (at half total dosage) separated by 1 h. The NC group was neither sham inoculated nor WTH challenged.

Lesion Scores and Body Weight Gain

All poults were weighed individually on days 0 and 14 for calculation of pre-challenge body weight gain (BWG). Presence of liver and cecal lesions associated with histomoniasis was recorded from all mortalities following WTH challenge. The individuals determining the lesion scores were blinded to the treatment groups. On day 13, all remaining poults were humanely euthanized by CO₂ asphyxiation and evaluated for the presence or absence of liver and cecal lesions associated with histomoniasis.

Statistical Analysis

In vitro data were analyzed using JMP Pro 14 software (SAS Institute Inc., Cary, NC) with significant differences between viable cells/mL in treatment groups determined using ANOVA. Pre-challenge BWG data were also analyzed using these methods. Where applicable, means were further separated using Tukey's multiple comparison post hoc test with values of $P \leq 0.05$ considered significant. Mortalities and presence of lesions related to histomoniasis were compared against the PC using Fisher's exact test and chi-square test with a difference of $P \leq 0.05$ considered significant.

RESULTS

***In vitro* Cell Viability Assays**

In assay 1, mean viable cells/mL (Log₁₀) for PBS, 0.4 mM DCA, 2 mM DCA, and 4 mM DCA treatments following 7 to 8.5 h incubation were 6.22, 6.25, 0.00, and 0.00, respectively (Table 2). The treatments of 2 and

4 mM DCA significantly reduced ($P \leq 0.05$) the concentration of viable cells as compared to either the PBS or the 0.4 mM DCA treatment.

In assay 2, mean viable cells/mL (Log₁₀) for PBS, 0.5 mM DCA, 1 mM DCA, 2 mM DCA, and 4 mM DCA treatments following 6 to 8 h incubation were 6.11, 6.12, 4.71, 0.00, and 0.00, respectively. The treatments of 1, 2, and 4 mM DCA significantly reduced ($P \leq 0.05$) the concentration of viable cells as compared to either the PBS or the 0.5 mM DCA treatment.

In assay 3, mean viable cells/mL (Log₁₀) for PBS, 0.5 mM DCA, 1 mM DCA, and 2 mM DCA following 4 to 6 h incubation were 6.30, 6.31, 6.16, and 1.34, respectively. The 1 mM DCA treatment significantly reduced ($P \leq 0.05$) the viable cells as compared to PBS or 0.5 mM DCA treatment. The 2 mM DCA treatment resulted in lower viable cells as compared to all other treatments. Following 27 to 29 h incubation, cells from assay 3 were enumerated with mean viable cells/mL (Log₁₀) counts of 6.48, 6.18, 4.46, and 0.00, respectively. The 0.5 mM DCA treatment resulted in lower ($P \leq 0.05$) viable cell counts as compared to the PBS. The 1 and 2 mM DCA treatment significantly reduced viable cells as compared to either the PBS or 0.5 mM DCA treatment.

Pre-challenge BWG From Day 0 to 14

The selected dietary concentrations of DCA had no statistically negative impact on day 0 to 14 pre-challenge BWG as compared to the basal control diet (Table 3). The 0.25% DCA diet group had higher ($P \leq 0.05$) pre-challenge BWG as compared to the 1% DCA diet group. No differences were observed in pre-challenge BWG in any of the DCA treatment groups as compared to the NC group.

Histomoniasis Infection Response and Lesions

Mortalities related to histomoniasis for the PC, 0.25% DCA, 0.5% DCA, and 1% DCA groups were 30, 50, 50, and 15%, respectively. Differences in mortalities associated with histomoniasis were not significant between any of the DCA treatment groups as compared to the PC group (Table 4). Liver lesions in the PC, 0.25% DCA, 0.5% DCA, and 1% DCA groups were present in 75, 95, 100, and 70%, respectively. Cecal lesions in the PC, 0.25% DCA, 0.5% DCA, and 1% DCA groups were present in 80, 95, 100, and 70%, respectively. The presence of liver and cecal lesions associated with histomoniasis was significantly higher ($P \leq 0.05$) in the 0.5% DCA diet group as compared to the PC group (Table 4). The NC group was negative for mortalities and lesions associated with histomoniasis.

Biliogenic Dietary Impact on Histomoniasis

Mortalities related to histomoniasis for the biliogenic diet group reached 35%. Liver and cecal lesion presence in the biliogenic diet group was 84.2 and 94.7%,

Table 1. Ingredient composition (% as-fed) of the biliogenic diet for induction of endogenous bile acids as compared to the control starter diet.

| Item | Control diet | Biliogenic diet ¹ |
|-------------------------------------|--------------|------------------------------|
| Corn | 49.3 | 45.7 |
| Soybean meal | 38.3 | 28.2 |
| Animal protein blend ² | 7.50 | 0.00 |
| Spray-dried egg powder ³ | 0.00 | 20.0 |
| Dicalcium phosphate | 1.41 | 2.75 |
| Limestone | 1.09 | 1.52 |
| Poultry fat | 1.03 | 1.00 |
| L-lysine HCl | 0.41 | 0.23 |
| Salt | 0.29 | 0.20 |
| DL-methionine | 0.39 | 0.15 |
| Vitamins and trace minerals | 0.15 | 0.15 |
| Choline chloride (60%) | 0.05 | 0.07 |
| L-threonine | 0.11 | 0.01 |

¹68 mg of biotin was added to the premix.

²Pro-Plus (H. J. Baker & Bro, Inc., Little Rock, AR).

³Spray-dried egg powder (Heartland Supply Co., Fayetteville, AR).

respectively. No statistical differences were detected in the day 0 to 14 pre-challenge BWG (Figure 2). Mortalities and lesions related to histomoniasis were not lesser in the biliogenic diet group as compared to the PC group (Table 5).

DISCUSSION

The in vitro evaluation of DCA at different time points and concentrations further confirmed that this compound markedly reduced *H. meleagridis* even following a shortened incubation time of 4 to 6 h. Inclusion of 4 mM DCA in both assays 1 and 2 further verified

Table 2. In vitro viability assays evaluating selected concentrations of DCA for antihistomonal properties.¹

| Assay 1 | Viable cells/mL (Log ₁₀) | |
|------------|--------------------------------------|--------------------------|
| Treatment | After 7–8.5 h incubation | |
| PBS | 6.22 ± 0.02 ^a | |
| 0.4 mM DCA | 6.25 ± 0.03 ^a | |
| 2 mM DCA | 0.00 ± 0.00 ^b | |
| 4 mM DCA | 0.00 ± 0.00 ^b | |
| Assay 2 | After 6–8 h incubation | |
| Treatment | After 6–8 h incubation | |
| PBS | 6.11 ± 0.02 ^a | |
| 0.5 mM DCA | 6.12 ± 0.05 ^a | |
| 1 mM DCA | 4.71 ± 0.25 ^b | |
| 2 mM DCA | 0.00 ± 0.00 ^b | |
| 4 mM DCA | 0.00 ± 0.00 ^b | |
| Assay 3 | After 4–6 h incubation | After 27–29 h incubation |
| Treatment | After 4–6 h incubation | After 27–29 h incubation |
| PBS | 6.30 ± 0.05 ^a | 6.48 ± 0.01 ^a |
| 0.5 mM DCA | 6.31 ± 0.03 ^a | 6.18 ± 0.07 ^b |
| 1 mM DCA | 6.16 ± 0.04 ^b | 4.46 ± 1.25 ^c |
| 2 mM DCA | 1.34 ± 0.92 ^c | 0.00 ± 0.00 ^c |

^{a–c}Data are expressed as mean ± SEM, n = 5 samples. Statistical evaluation using ANOVA followed by Tukey's multiple post hoc test. Means with no common superscript differ significantly ($P \leq 0.05$).

Abbreviation: DCA, deoxycholic acid.

¹Assays 1–3 began with concentrations of 2.01×10^6 , 6.88×10^5 , and 6.35×10^5 cells/mL of the wild-type *Histomonas meleagridis*, respectively, added at a ratio of 100 μ L cells:50 μ L treatment and incubated under anaerobic conditions at 40°C.

the toxicity of DCA following 2 different incubation time points. Viability of *H. meleagridis* was effectively reduced in vitro by DCA; viable cell counts decreased with increased concentration of DCA. Inhibition of *H. meleagridis* is known to occur with changes in pH in vitro (Hauck et al., 2010). However, changes in pH were not measured in the in vitro part of this experiment due to xenic bacteria growing with this *H. meleagridis*, leading to marked swings in pH change within the culture media. If antihistomonal properties of DCA are conferred in vivo, use as a commercial feed additive might be more accepted since DCA is naturally occurring and is regenerated during enterohepatic recirculation. Dietary inclusion of DCA at the selected concentrations neither prevented nor mitigated the disease in turkeys challenged with WTH. The presence of more lesions within the 0.5% diet DCA group as compared to the PC group further contributes to the conclusion from this study that DCA was not effective against histomoniasis under these experimental conditions. Considerations of inclusion of higher dietary concentrations of DCA are not encouraging given that we were apparently approaching toxic levels and inclusion higher than 10 kg/ton would likely not be practical.

Cholesterol, an important precursor for bile acids, has recently been shown to enhance *H. meleagridis* growth in vitro (Gruber et al., 2018). Based on this recent finding by Gruber et al. (2018), the biliogenic diet formulation, which included 20% whole egg powder, may have increased normal enteric cholesterol levels and inadvertently aided the parasite. Both lecithin-enriched and egg-enriched diets have been associated with increased

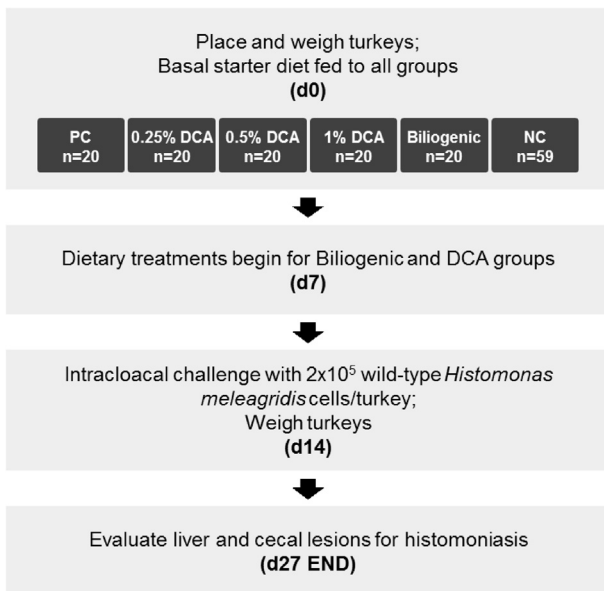


Figure 1. In vivo trial experimental timeline evaluating different dietary concentrations of DCA and a biliogenic diet as a preventative means against histomoniasis in turkeys. The biliogenic diet was formulated with 20% whole egg powder to encourage endogenous bile acid production. Both the NC and PC groups received the basal starter diet for the duration of the experiment. Lesions were evaluated based on the presence or absence of “target-like” liver lesions and cecal cores characteristic of histomoniasis. Abbreviations: DCA, deoxycholic acid; NC, non-challenged control; PC, positive-challenged control.

Table 3. Effect of dietary inclusion of selected concentrations of DCA on day 0 to 14 BWG during pre-challenge phase.

| Treatment | Day 0 to 14 pre-challenge BWG (g) ¹ |
|------------------------------------|--|
| Positive-challenged control day 14 | 270 ± 9.36 ^{a,b} |
| 0.25% DCA | 289 ± 14.0 ^a |
| 0.5% DCA | 257 ± 13.1 ^{a,b} |
| 1% DCA | 242 ± 12.4 ^b |
| Non-challenged control | 265 ± 6.34 ^{a,b} |

^{a,b}Means ± SEM with no common superscript differ significantly ($P \leq 0.05$).

Abbreviations: BWG, body weight gain; DCA, deoxycholic acid.

¹Statistical evaluation using ANOVA followed by Tukey's multiple post hoc test.

total bile acid output (Lindsay et al., 1969; LeBlanc et al., 1998; Yang et al., 2012). The classical pathway for bile acid synthesis contains cholesterol 7 α -hydroxylase (CYP7A1) and contributes to approximately 75% of bile acid synthesis within the liver (Lefebvre et al., 2009). CYP7A1, the rate-limiting enzyme for bile acid synthesis from cholesterol, resulted in increased mRNA expression within egg yolk (31.25%) and whole egg powder (55.56%)-enriched diets as compared to cholesterol (0.75%) and standard control diets (Yang et al., 2012). This increased mRNA expression indicates that the egg-enriched diets upregulated the CYP7A1 activity, thereby promoting bile acid synthesis and possibly contributing to the increased bile acid output; however, enzyme expression was not evaluated in our current study. Since bile acid levels were not evaluated in the present study, the putative biliogenic diet formulation used was not confirmed to have an effect on bile acid output. Future studies should include evaluation of bile acid production following proposed chemoprophylaxis treatments. Moreover, the rate of cecal retrograde of the dietary inclusion may not have been suitable for the proposed chemoprophylaxis agents to reach the target area of *H. meleagridis* reproduction. Other limitations include the possibility that DCA was further metabolized and neutralized within the gastrointestinal tract and not available to be retrograded in a form that is toxic to *H. meleagridis*.

Although DCA treatment resulted in a significant reduction of cells in vitro, the ineffectiveness to prevent histomoniasis in vivo is consistent with the variable results of other candidates previously evaluated against

this disease. With the absence of approved and effective compounds for the prevention or treatment of histomoniasis, the contribution of negative data such as these is important to ensure that similar experiments are not conducted with DCA. Extracts of the medicinal herb *Artemisia annua* as well as artemisinin (a main active compound) exhibited antihistomonal properties in vitro but failed to prevent *H. meleagridis* infection within experimentally challenged turkeys and chickens (Thøfner et al., 2012). In vitro susceptibility did not translate to in vivo results. These findings are similar to our DCA treatment results, further emphasizing the importance of incorporating in vivo evaluation of antihistomonal chemoprophylaxis compounds rather than relying only upon positive in vitro data. Further confounding this already complicated disease, the cecal environment wherein *H. meleagridis* resides is constantly in a state of flux, demonstrating the difficulties in controlling histomoniasis. Bacterial flora contribute an important role in disease development; therefore, the influence of chemoprophylaxis candidates on the bacteria located in the gastrointestinal environment as well as the impacts on *H. meleagridis* should be considered (Callait et al., 2002; McDougald, 2005). In conclusion, DCA and the formulated biliogenic diet were evaluated for the first known time against histomoniasis, but none of the dietary treatments, as administered in this study, were effective in the mitigation of disease in vivo.

Table 4. Effect of dietary inclusion of selected concentrations of DCA on mortality and lesions related to histomoniasis.^{1,2}

| Treatment | Mortality | Liver lesions | Cecal lesions |
|-----------------------------|-----------|----------------------|--------------------|
| Positive-challenged control | 6/20 | 15/20 | 16/20 |
| 0.25% DCA | 10/20 | 19/20 | 19/20 |
| 0.5% DCA | 10/20 | 20/20 ^{†,*} | 20/20 [*] |
| 1% DCA | 3/20 | 14/20 | 14/20 |

Abbreviation: DCA, deoxycholic acid.

¹Post-challenge cumulative mortality and classical lesions associated with histomoniasis from day 9 post-challenge until day 13 experiment termination. Lesions were determined based on the presence or absence of classic histomoniasis characterized by target-like liver lesions and cecal cores. No lesions or mortalities as a result of histomoniasis occurred within the non-challenged control group (0/59).

²Statistical evaluation used the chi-square test (indicated by “*”) and Fisher's exact test (indicated by “†”) as compared to the positive-challenged control with significance at $P \leq 0.05$.

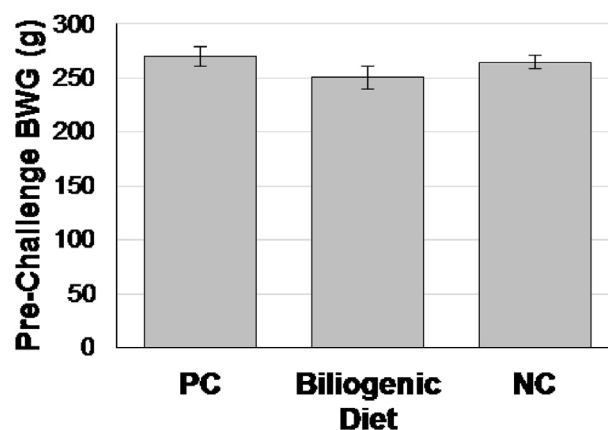
**Figure 2.** Effect of putative biliogenic diet on day 0 to 14 BWG during pre-challenge phase. No significant differences were observed with ANOVA ($P \leq 0.05$). The biliogenic diet was formulated with 20% whole egg powder. Abbreviations: BWG, body weight gain; NC, non-challenged control; PC, positive-challenged control.

Table 5. Effect of a putative biliogenic diet on mortality and lesions related to histomoniasis.^{1,2}

| Treatment | Mortality | Liver lesions | Cecal lesions |
|------------------------------|-----------|---------------|---------------|
| Positive-challenged control | 6/20 | 15/20 | 16/20 |
| Biliogenic diet ³ | 7/20 | 16/19 | 18/19 |

¹Post-challenge cumulative mortality and classical lesions associated with histomoniasis from day 6 post-challenge until day 13 experiment termination. Lesions were determined based on the presence or absence of classic histomoniasis characterized by target-like liver lesions and cecal cores. No lesions or mortalities as a result of histomoniasis occurred within the non-challenged control group (0/59).

²Statistical evaluation used the chi-square test and Fisher's exact test with significance at $P \leq 0.05$. No significant differences in mortalities or lesions were observed.

³The biliogenic diet was formulated with 20% whole egg powder. One poult was excluded from the lesion data set for the biliogenic diet group due to tabulation error.

ACKNOWLEDGMENTS

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

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