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Caprylic Acid Reduces Enteric *Campylobacter* Colonization in Market-Aged Broiler Chickens but Does Not Appear To Alter Cecal Microbial Populations†

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ABSTRACT

Campylobacter is a leading cause of foodborne illness in the United States, and epidemiological evidence indicates poultry products to be a significant source of human *Campylobacter* infections. Caprylic acid, an eight-carbon medium-chain fatty acid, reduces *Campylobacter* colonization in chickens. How caprylic acid reduces *Campylobacter* carriage may be related to changes in intestinal microflora. To evaluate this possibility, cecal microbial populations were evaluated with denaturing gradient gel electrophoresis from market-age broiler chickens fed caprylic acid. In the first trial, chicks ($n = 40$ per trial) were assigned to four treatment groups ($n = 10$ birds per treatment group): positive controls (*Campylobacter*, no caprylic acid), with or without a 12-h feed withdrawal before slaughter; and 0.7% caprylic acid supplemented in feed for the last 3 days of the trial, with or without a 12-h feed withdrawal before slaughter. Treatments were similar for trial 2, except caprylic acid was supplemented for the last 7 days of the trial. At age 14 days, chicks were orally challenged with *Campylobacter jejuni,* and on day 42, ceca were collected for denaturing gradient gel electrophoresis and *Campylobacter* analysis. Caprylic acid supplemented for 3 or 7 days at 0.7% reduced *Campylobacter* compared with the positive controls, except for the 7-day treatment with a 12-h feed withdrawal period. Denaturing gradient gel electrophoresis profiles of the cecal content showed very limited differences in microbial populations. The results of this study indicate that caprylic acid's ability to reduce *Campylobacter* does not appear to be due to changes in cecal microflora.

Campylobacter jejuni is one of the leading causes of human foodborne illness in the United States, and epidemiological evidence indicates poultry and poultry products as significant sources of human *Campylobacter* infection *(10, 15, 35)*. Colonization of poultry by *Campylobacter* is widespread and difficult to prevent even with proper biosecurity measures *(6, 25, 54)*.

Cecal carriage of *C. jejuni* results in horizontal transmission of the pathogen and carcass contamination during processing. Therefore, antemortem intervention strategies to reduce *C. jejuni* counts in the chicken intestinal tract are critical for delivering a microbiologically safer product. Caprylic acid, a medium-chain fatty acid with eight carbons, is naturally found in breast milk, bovine milk *(19),* and coconut oil *(20, 48)*. It is a food-grade compound classified as generally recognized as safe by the U.S. Food and Drug Administration. Caprylic acid has been reported to be effective in killing a variety of bacterial pathogens, including *Salmonella* Enteritidis in chicken cecal contents *(53), Escherichia coli* O157:H7 in bovine rumen fluid *(2),* and *Staphylococcus aureus* in bovine milk *(33)*. Caprylic acid has also shown efficacy against *Clostridium perfringens* and *E. coli* in rabbits *(43, 44)*. Thormar and coworkers *(51)* reported that monocaprin, the monoglyceride of capric acid ($C_{10:0}$), was effective in killing significant populations of *C. jejuni* in chicken feed. Recently, we have demonstrated the prophylactic efficacy of feed supplemented with caprylic acid against *C. jejuni* in 10-day-old broiler chicks *(45)* and therapeutic efficacy against *Campylobacter* in 15-day-old and market-aged (42 days old) broiler chickens *(46, 47)*. However, the mechanism of action of caprylic acid against *Campylobacter* has not been established.

It is possible caprylic acid alters the intestinal microflora, resulting in a competitive disadvantage for *Campylobacter*. This idea is supported by results demonstrating changes in intestinal bacterial population in poultry by using different feeds and feed additives *(3, 16, 17)*. This is also one of the theories on how probiotics adversely affect enteric *Salmonella* colonization *(27, 28, 30, 49)*. To test this possibility, we conducted two trials to determine any

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changes in cecal microbial populations by using denaturing gradient gel electrophoresis (DGGE) in market-age broiler chickens fed caprylic acid. The ceca were evaluated because this is the area of the intestine with the highest concentration of *Campylobacter* in poultry *(6, 9)*. The 0.7% caprylic acid dose was used because it has shown to have the most consistent efficacy against *Campylobacter (45–47)*.

MATERIALS AND METHODS

Experimental design. Two trials were conducted in which day-of-hatch chicks ($n = 40$ per trial) were assigned to four treatment groups ($n = 10$ birds per treatment group): positive controls (*Campylobacter*, no caprylic acid), with or without a 12-h feed withdrawal before slaughter; and 0.7% caprylic acid supplemented in finisher feed for the last 3 days of the trial, with or without a 12-h feed withdrawal before slaughter. Treatments were similar for trial 2, except caprylic acid was fed for the last 7 days of the trial. At 42 days of age, chickens were euthanized by CO_2, and cecal contents were collected for *Campylobacter* enumeration and DNA extraction for DGGE analysis. Birds from each treatment group were weighed on day 42, and the feed was weighed prior to and immediately after the 3- or 7-day dosing period.

Bacterial strains and dose. A five-strain mixture of wild-type *C. jejuni* isolated from chickens was used to colonize the birds as previously described *(14)*. In short, 10-μl loops of each frozen strain was cultured into 5 ml of *Campylobacter* enrichment broth and incubated for 48 h at 42°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). For the second passage, again 10-μl loops from each strain were transferred into 5 ml of fresh *Campylobacter* enrichment broth to be incubated for 24 h at 42°C. After incubation, equal volumes of the cultures were pooled in a 25-ml tube, and 3-ml portions were transferred into a glass tube to be read in a spectrophotometer to determine the approximate bacterial population present in the five-strain mixture. After measuring the absorbance (optical density), the tube containing the culture was centrifuged at 3,500 $\times$ *g* for 10 min. The supernatant was discarded, and the remaining pellet was resuspended with the same volume of Butterfield's phosphate diluent. At 14 days of age, seeder chicks ($n = 5$ per treatment group) were orally challenged with 250 μl of inoculum by using a 10-ml syringe connected to a stainless steel and sterilized cannula. To determine the concentration of the culture used to challenge the birds, the inoculum was quantitated by serially diluting on *Campylobacter* line agar *(24)* plates incubated for 48 h at 42°C under microaerophilic conditions (2.6×10^8 CFU/ml for both trials).

Cecal *Campylobacter* determination. The ceca of the chicken are composed of two identical ceca *(11, 26)*. One cecum was collected from 10 chickens per treatment for *Campylobacter* enumeration and the other cecum for DNA extraction (see below). Cecal *Campylobacter* content was enumerated by the procedure of Cole and coworkers *(12)*. Briefly, a cecum from each bird was transferred to a sterile plastic bag. The contents were squeezed into 15-ml tubes, serially diluted (1:10) with Butterfield's phosphate diluent, and then inoculated onto labeled *Campylobacter* line agar plates. Labeled *Campylobacter* line agar plates were incubated for 48 h at 42°C under microaerophilic conditions. Direct bacterial counts were recorded and converted to CFU per gram of the cecal content. *Campylobacter* colonies were confirmed by a latex agglutination test (PANBIO, Inc., Columbia, MD) and further identified as *C. jejuni* isolates by using API Campy (bioMérieux, Hazelwood, MO).

DNA extraction. Five birds per treatment were randomly chosen for cecal DNA extraction according the procedure of Hume and coworkers *(18)*. Digesta from individual cecum samples were prepared separately to obtain 50 ng of DNA per bird, and then samples were pooled for each treatment group to obtain 250 ng of DNA for DGGE analysis. Prior to pooling, the individual samples were prepared by adding 0.5 g of digesta to 4.5 ml of sterile Butterfield phosphate diluent, vortexed, and 1 ml of this mixture was centrifuged in a microfuge tube at 8,000 $\times$ *g* for 10 min. The centrifugation supernatant was aspirated and discarded. The pellets were used to extract DNA immediately by using the Aqua Pure Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA). Genomic DNA was isolated from 1 ml of each sample according to the method described in the kit. Briefly, samples were centrifuged at 14,000 $\times$ *g* for 15 s to pellet cells. The supernatant was carefully removed with a pipette and 300 μl of genomic DNA lysis solution was added and incubated at 80°C for 5 min.

RNase treatment was performed by adding 1.5 μl of RNase A solution (4 mg/ml) to the cell lysate, and then samples were mixed and incubated at 37°C for 45 min. The protein was precipitated by adding 100 μl of protein precipitation solution, vortexed vigorously at high speed for 20 s, and then centrifuged at 14,000 $\times$ *g* for 3 min. The supernatant was poured off, leaving behind the precipitated protein pellet, into a clean, 1.5-ml microfuge tube containing 300 μl of 100% isopropanol (2-propanol). After adding glycogen as a DNA carrier (0.5 μl of 20 mg/ml glycogen per 300 μl of isopropanol), samples were mixed and centrifuged at 14,000 $\times$ *g* for 1 min. The supernatant was then poured off, 300 μl of 70% ethanol was added, the tubes were inverted several times to wash the DNA pellet, and then the tubes were centrifuged at 14,000 $\times$ *g* for 1 min. The ethanol was carefully poured off, and the tubes were inverted and drained. The DNA was hydrated by adding 50 μl of DNA hydration solution and incubated at 65°C for 5 min. Finally, the samples were vortexed for 5 s at medium speed, and briefly pulse spun to collect sample at the bottom of the tube, and the samples were stored at −20°C for later use.

PCR amplification. Each PCR reaction contained 250 ng of pooled DNA from a treatment of five chickens (50 ng per chicken) *(18)*, JumpStart REDTaq Ready Mix (Sigma Aldrich, St. Louis, MO), 50 pmol of each a forward, and a reverse primer. The reverse primer had a 40-bp G–C clamp to curtail migration of the denatured strand in the denaturing gel *(32, 42)* and 5% (wt/vol) acetamide to eliminate nonpreferential annealing *(38)*. The reaction was conducted in a PTC-200 Peltier thermal cycler by using the following program: (i) initial denaturation at 94.9°C for 2 min, (ii) subsequent denaturation at 94°C for 1 min, (iii) annealing at 67°C for 45s (0.5°C per cycle to a touchdown temperature of 58°C), and (iv) extension at 72°C for 2 min. The remainder of the program consisted of (i) 17 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 45 s, and final extension at 72°C for 2 min; (ii) denaturation at 94°C for 1 min; (iii) annealing at 58°C for 45 s; (iv) repeating the denaturation at 94°C for 1 min and the annealing at 58°C for 45 s during 12 cycles; (v) extension at 72°C for 7 min; and (vi) 4°C for the final temperature.

Electrophoresis conditions. The 8% polyacrylamide gels had (acrylamide/bisacrylamide ratio of 37.5:1), a 35 to 60% urea deionized formamide gradient created by diluting a 100%

denaturing acrylamide (7 M urea plus 40% deionized formamide). Amplified DNA samples were added to the same volume of 2× loading buffer containing bromophenol blue (0.05% [wt/vol]), xylene cyanol (0.05% [wt/vol]), and glycerol (70% [vol/vol]); 4 μl of this solution was placed into each sample well (16-well comb). A universal mutation detection system for electrophoresis was used to run the gel in 0.5× triethanolamine (20 mM Tris [pH 7.4], 10 mM sodium acetate, and 0.5 M EDTA) at 59°C for 17 h at 60 V. After running, the gel was stained with diluted SYBR Green 1 (1:10,000). Molecular Analyst Fingerprinting Software, version 1.6 (Bio-Rad), was used for determining fragment pattern relatedness, which is based on the Dice similarity coefficient and the unweighted pair group arithmetic mean method by using arithmetic averages for clustering. The Dice coefficient, which ranges from 0 to 1, determines the degree by which the patterns are alike. By sequentially comparing the patterns and the construction of a relatedness tree (dendrogram), the cluster groups were determined. The dendrogram reflects the relative similarity, which is related to closeness or grouping. The relative similarity is indicated by the percentage similarity coefficient bar located above each dendrogram.

Statistical analysis. Data were analyzed by analysis of variance by using the GLM procedure of SAS *(41)*. The numbers of *Campylobacter* colonies were logarithmically transformed (log CFU per gram) before analysis to achieve homogeneity of variance *(7)*. Treatment means were partitioned by least-squares means analysis *(41)*. A probability of <0.05 was required for statistical significance.

RESULTS

***Campylobacter* concentration.** The supplementation of caprylic acid at 0.7% in the feed for the last 3 days of the growing period reduced ($P < 0.05$) *Campylobacter* counts in the cecal contents of market-aged chickens, with or without a 12-h feed withdrawal, when compared with positive-control treatments (Fig. 1A). When 0.7% caprylic acid was feed for 7 days, *Campylobacter* was reduced ($P < 0.05$) for birds without feed withdrawal, but not for those with a 12-h feed withdrawal period (Fig. 1B).

Cecal DGGE profile, body weight, and feed consumption. The DGGE profiles of the cecal microbial community are shown in Figure 2A through 2D. The birds fed 0.7% caprylic acid for 3 days had 87.8% (Fig. 2A) or 88.3% (Fig. 2B) similarity for the birds without or with a 12-h feed withdrawal period when compared with the positive controls, respectively. Similarly, when birds were fed caprylic acid for 7 days, they had 96.1% (Fig. 2C) or 97.5% (Fig. 2D) similarity to positive controls, without or with a feed withdrawal period, respectively. Body weight and feed consumption were not affected by caprylic acid treatments in any of the experiments (Table 1).

DISCUSSION

Therapeutic supplementation of caprylic acid at 0.7% in the feed for 3 or 7 days before slaughter showed a consistent 3- to 4-log reduction in cecal *C. jejuni* counts in market-aged broiler chickens. When a 12-h feed withdrawal period was evaluated, the 0.7% dose for 3 days but not 7 days produced approximately a 3-log reduction in *C. jejuni* counts. These results are consistent with previous reports from our laboratory *(45–47)*.

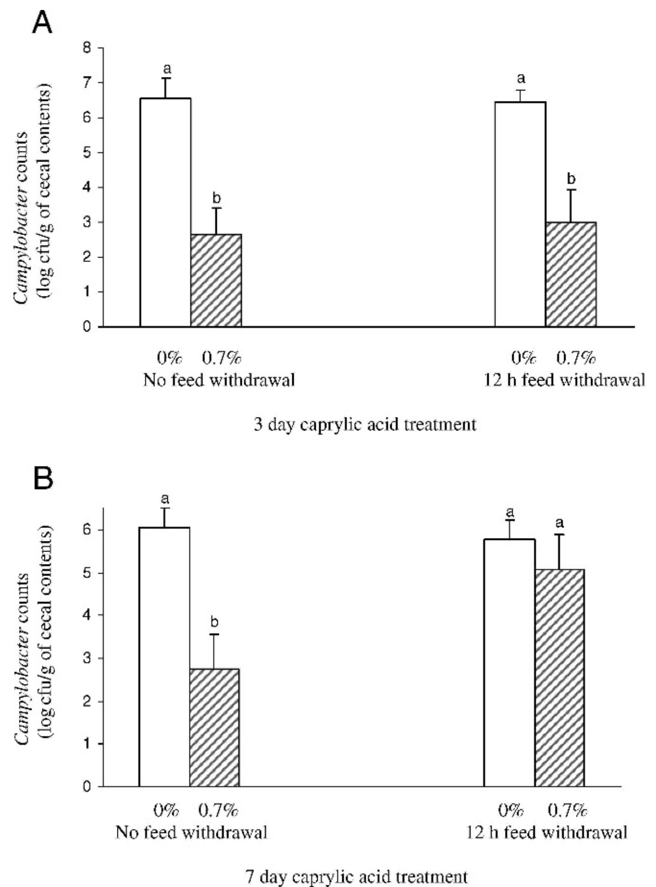

FIGURE 1. *Cecal* Campylobacter jejuni *counts (means ± SEM) in 42-day-old broiler chickens (*n *= 10 birds per treatment) with or without a 12-h feed withdrawal before necropsy. Caprylic acid was added to the feed for 3 days (A) or 7 days (B) before necropsy. Treatments with different letters denote significant (*P *< 0.05) difference.*

In an effort to understand how caprylic acid reduces cecal *Campylobacter* counts, intestinal microbial populations were evaluated by DGGE analysis. Denaturing gradient gel electrophoresis is a PCR-based technique that has been used to study changes in the chicken intestinal microbial population communities *(17, 21)*. DGGE is capable of profiling the predominant microbes of the intestine, and it is thought to pick up those microbes representing >1% of the entire population of microbes *(22, 31, 32)*. In this study, changes in poultry digestive microbial populations were observed as differences in amplicon patterns on denaturing gradient gels after amplification of bacterial 16S rDNA. DGGE results are presented as percent similarity coefficients of the cecal microbial populations. The results from the current study indicate that caprylic acid had little, if any effect on the cecal microbial community. Population similarities were approximately 88 or 96% between the birds fed caprylic acid for 3 or 7 days versus the non–caprylic acid-fed *Campylobacter*-positive controls (Fig. 2A through 2D). It is interesting that there is little difference between the population percent similarity of the birds fed 0.7% caprylic acid for 7 days without or with feed

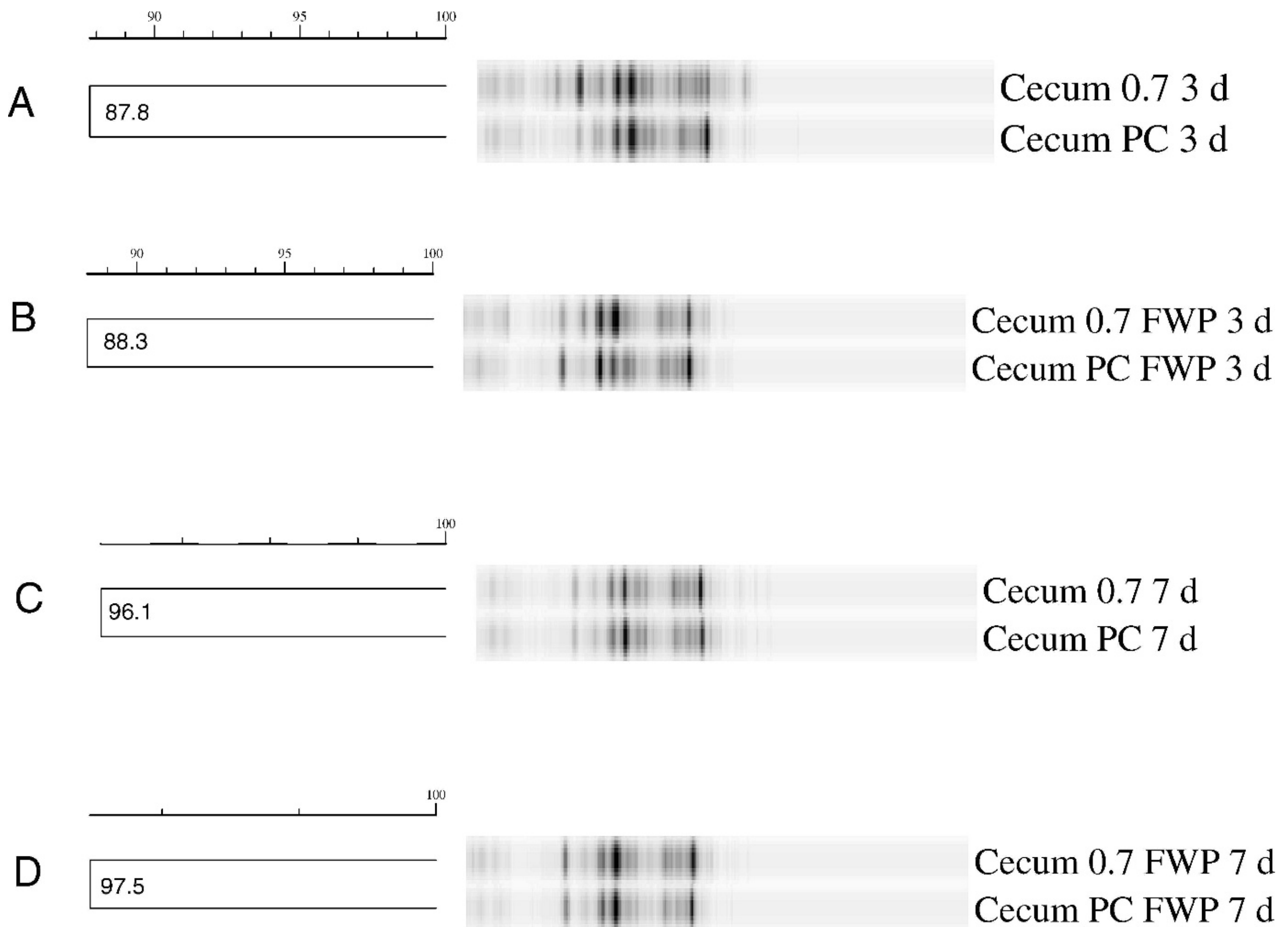

FIGURE 2. *DDGE generated from cecal contents of 42-day-old chickens. (A) Cecum 0.7 3 d (0.7% caprylic acid supplemented in feed for 3 days), cecum PC 3 d (3-day positive controls); (B) cecum 0.7 FWP 3 d (0.7% caprylic acid supplemented in feed for 3 days, with a 12-h feed withdrawal period), cecum PC FWP 3 d (3-day positive controls with a 12-h feed withdrawal period); (C) cecum 0.7 7 d (0.7% caprylic acid supplemented in feed for 7 days), cecum PC 7 d (7-day positive controls); (D) cecum 0.7 FWP 7 d (0.7% caprylic acid supplemented in feed for 7 days with a 12-h feed withdrawal period), cecum PC FWP 7 d (7-day positive controls with a 12-h feed withdrawal).*

withdrawal (96.1 versus 97.5%). Yet, *Campylobacter* counts were reduced by approximately 3 log in birds without feed withdrawal as opposed to no differences in those with a 12-h feed withdrawal. These results suggest that the mechanism of action of caprylic acid is not related to changes in intestinal microbial populations. It is also possible that minute changes in the numbers of specific bacteria (and their byproducts, e.g., volatile fatty acids) in

TABLE 1. *Effect of caprylic acid on body weight and feed consumption of market-aged broiler chickens*

Treatment	3-Day treatment		7-Day treatment	
	Body wt (g)[a]	Feed consumption (g)[b]	Body wt (g)	Feed consumption (g)
No feed withdrawal				
Positive controls[c]	2,850.0 ± 151.0 A[d]	494 A	2,637.7 ± 169.3 A	1,146 A
0.7% Caprylic acid	3,007.2 ± 117.8 A	496 A	2,974.1 ± 60.2 A	1,158 A
Feed withdrawal				
Positive controls	2,803.0 ± 139.4 A	504 A	2,769.3 ± 91.8 A	1,126 A
0.7% Caprylic acid	2,762.0 ± 77.4 A	490 A	2,607.0 ± 128.0 A	1,178 A

[a] Body wt, the average body weight per bird per treatment ($n = 10$ per treatment group) for the entire 42-day study period.
[b] Feed consumption represents the average feed consumption per treatment per bird for the 3- or 7-day treatment period prior to necropsy.
[c] Positive controls were challenged with *Campylobacter* (see ''Material and Methods'') but did not receive caprylic acid in the diet.
[d] Means within columns within feed withdrawal treatment with no common letter differ significantly ($P < 0.05$).

the cecal population may also be influencing *Campylobacter* counts. At this time, however, such changes are beyond the capabilities for accurate detection by DGGE.

Results from the current study and previous studies from our laboratory suggest the possibility that caprylic acid has a direct inhibitory effect on *Campylobacter*. Previous research from our laboratory demonstrated a physical alteration of the gastrointestinal tract (reduced crypt depth and goblet cell numbers) consistent with a reduction in *Campylobacter* content after bacteriocin treatment *(12)*. As *Campylobacter* preferentially colonizes the mucus produced by goblet cells in the cecal crypts of poultry *(4, 29)*, the bacteriocins mode of action appears to be indirect, i.e., altering intestinal niches used by *Campylobacter* for colonization. Dosing with caprylic acid, however, did not produce similar indirect effects *(46)*. It would also appear that a decrease in intestinal pH is also not responsible for caprylic acid–mediated *C. jejuni* reduction, since its treatment had limited, if any, effect on cecal pH *(47)*. Since fatty acids exert a direct effect on bacterial biological membranes *(5)*, it is hypothesized that caprylic acid may compromise the outer membrane determinants in *Campylobacter*, which are needed for bacterial adaptation to host environment and colonization. It is also possible that caprylic acid has a direct inhibitory effect on the expression of virulence factors necessary of *C. jejuni* colonization in chicks. For example, medium-chain fatty acids have been found to significantly reduce the invasiveness of *Salmonella* Enteritidis in invasion assays involving a human colon carcinoma (T84) cell line *(52)*. These fatty acids were found to suppress the expression of *hilA*, a regulator of virulence genes located in the *Salmonella* pathogenicity island I, which is directly involved in the invasion of intestinal epithelial cells. However, additional research is necessary to confirm these hypotheses.

Caprylic acid being a natural and relatively inexpensive compound may represent a practical and economical strategy for poultry farmers for reducing *C. jejuni* carriage in chickens. In the present study, the 0.7% dose of caprylic acid therapeutically reduced cecal *C. jejuni* counts of market-aged broiler chickens by approximately 3 to 4 log. This reduction could have significant beneficial implications on food safety, since, during processing, enteric contents can contaminate the carcass, thereby resulting in foodborne transmission of *C. jejuni (1, 40)*. It has been estimated that a 2-log reduction in *C. jejuni* populations on poultry carcass contaminations could produce up to a 30-fold reduction in human c*ampylobacteriosis* cases *(23, 39)*. Furthermore, this treatment, consistent with our previous studies *(46, 47)*, did not have any effect on body weight or feed consumption.

Caprylic acid was also effective after a 12-h feed withdrawal when fed for 3 days. Feed withdrawal is a common industry practice to reduce intestinal digesta and intestinal rupture, thereby reducing the probability of carcass contamination *(34, 36, 50)*. Unfortunately, feed withdrawal can be associated with increased pecking of manure-contaminated litter, which may increase the amount of pathogens in the intestine of the chickens *(8, 13, 37, 50)*. Therefore, dosing with 0.7% caprylic acid in the feed for 3 days allows the use and benefits of a 12-h feed withdrawal in addition to reducing cecal *Campylobacter* counts.

The results of the present study demonstrate that 0.7% caprylic acid supplemented in the feed 3 or 7 days prior to slaughter can reduce cecal *Campylobacter* counts by approximately 3 to 4 log. This effect is apparently not associated with changes in microbial populations. It appears that caprylic acid has a direct inhibitory effect on enteric *Campylobacter* in poultry.

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