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Inclusion of trans-cinnamaldehyde and caprylic acid in feed results in detectable concentrations in the chicken gut and reduces foodborne pathogen carriage

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ABSTRACT Poultry act as a major reservoir host for *Salmonella* and *Campylobacter* spp., the 2 leading causes of foodborne illnesses globally and in the United States. Preharvest stage interventions to reduce foodborne pathogen carriage in poultry are increasingly informed by consumer preference for antibiotic-free poultry production. The in-feed inclusion of plant-derived antimicrobial compounds is a promising antibiotic alternative strategy to reduce foodborne pathogen load in the broiler chicken gut. Yet, the fate of these phytochemicals through the broiler chicken gastrointestinal tract is unknown. Likewise, while in-feed phytochemicals have been widely demonstrated in challenge models to reduce foodborne pathogen carriage, little is known regarding efficacy to curb natural routes of infection. As such, the aim of the present study was 2-fold. We sought to determine the concentrations of 2 phytochemicals, trans-cinnamaldehyde and caprylic acid, in each region of the chicken gastrointestinal tract following their in-feed inclusion over a 6-wk production period. In addition, we investigated how the in-feed provision of these phytochemicals may protect against environmental acquisition of *Campylobacter jejuni* and *Salmonella* spp. Trans-cinnamaldehyde and caprylic acid were detected in crop, gizzard, duodenal, jejunal, and ileal contents. Crop and gizzard concentrations were not significantly ($P > 0.05$) different. A significant ($P < 0.05$) decrease in phytochemical concentration was observed in intestinal regions compared to crop and gizzard. Trans-cinnamaldehyde was consistently identified in cecal and colon contents, while caprylic acid was not detectable in these regions. Trans-cinnamaldehyde and caprylic acid were found to reduce ($P < 0.05$) *Salmonella* load. Together, our data establish that the in-feed addition of trans-cinnamaldehyde and caprylic acid, 2 phytochemicals that have previously been shown to exert antimicrobial activity against poultry-associated foodborne pathogens, results in detectable concentrations in the broiler chicken gastrointestinal tract. By providing researchers with a gastrointestinal region-by-region map of phytochemical concentrations, the present study is expected to inform the choice of in-feed phytochemicals targeting foodborne pathogen carriage in the broiler chicken gastrointestinal tract.

Key words: phytochemical, chicken, gut, feed, foodborne pathogen

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INTRODUCTION

Poultry is both the most consumed animal protein in the world (Whitton et al., 2021) and a major reservoir of the human disease-causing foodborne pathogens *Campylobacter jejuni* and *Salmonella* spp. (Scharff, 2020). Antibiotic stewardship and consumer preference for antibiotic-free poultry production have driven the global call for the antibiotic-alternative management of poultry foodborne pathogen carriage. Plant-derived antimicrobial compounds are one such antibiotic alternative strategy utilized in both pre- and postharvest poultry production. During the preharvest stage, phytochemicals are often delivered in-feed to reduce intestinal populations of foodborne pathogens as well as effect complementary outcomes such as the promotion of bird growth (Venkitanarayanan et al., 2013; Lillehoj et al., 2018). Trans-cinnamaldehyde and caprylic acid are 2 such phytochemicals that have shown efficacy in reducing foodborne pathogen carriage in broilers (Solis de Los Santos et al., 2008a,b; Kollanoor-Johny et al., 2012a), vertical transmission in layer chickens (Upadhyaya et

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al., 2015), as well as in postharvest settings (Allen et al., 2023). While in vitro studies have revealed direct effects of these phytochemicals on bacterial cells (Upadhyay et al., 2017; Wagle et al., 2019), it is unknown whether these phytochemicals, when consumed in feed, reach the chicken's ceca and other lower intestinal regions which are the predominant sites of foodborne pathogen colonization (Beery et al., 1988; Meade et al., 2009).

The use of organic acids and essential oils during the preharvest stage of poultry production is widespread (Micciche et al., 2019; Peh et al., 2020). Caprylic acid, an organic acid, is a medium chain fatty acid that naturally occurs in a variety of foodstuffs including coconut and palm kernel oils as well as cow's milk. Likewise, trans-cinnamaldehyde was originally isolated from cinnamon tree bark (*Cinnamomum zeylandicum*) (Upadhyay et al., 2017). As generally recognized as safe (**GRAS**) compounds by the US Food and Drug Administration (**FDA**), trans-cinnamaldehyde and caprylic acid are approved for addition in food products. Trans-cinnamaldehyde and other phytochemicals have also been approved for use in the European Union as feed additives in broiler chicken feed (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2015). While these phytochemicals are therefore available to poultry producers, the lack of information pertaining to their survivability through the chicken gastrointestinal tract likely hinders their widespread adoption in preharvest production. Indeed, it was previously reported that trans-cinnamaldehyde is rapidly absorbed in the porcine upper gastrointestinal tract (Michiels et al., 2008) yet no investigation to date has reported whether phytochemicals provided in feed later appear, and at what concentrations, in the chicken gastrointestinal tract.

As such, the present study sought to determine whether chickens fed a diet containing either trans-cinnamaldehyde or caprylic acid results in detectable concentrations in each region of the gastrointestinal tract, and to investigate if these diets reduce carriage of *Salmonella* and *Campylobacter* spp.

MATERIALS AND METHODS

Chickens and Diets

All procedures and management practices were approved by the University of Arkansas Division of Agriculture Institutional Animal Care and Use Committee (**IACUC** protocol# 22037) before beginning the study. Day-of-hatch broiler chicks were acquired from a local hatchery (Cobb, Siloam Springs, AR). All chicks were weighed, tagged, and then randomly allocated to control (corn/soy diet), trans-cinnamaldehyde (control group feed supplemented with 0.75% trans-cinnamaldehyde), or caprylic acid (control group feed supplemented with 0.75% caprylic acid) diet groups. All chicks were placed into floor pens containing fresh pine shavings at a stocking density of 40 chicks per pen (2 replicate pens per group) and provided ad libitum feed and water throughout the study period (42 d).

Sample Collection

Cloacal swabs were collected randomly from 20 birds at the beginning of the study and tested for the presence or absence of *Salmonella* or *Campylobacter* in day of hatch birds. Fresh fecal samples were collected weekly from each quarter of the pen. The 4 samples from each pen were pooled, serially diluted (1:10) with buffered phosphate diluent (**BPD**) and plated on XLD Agar for *Salmonella* and *Campylobacter*-Line Agar (**CLA**) plates for *Campylobacter*. The XLD Plates were incubated at 37°C for 24 h, and the CLA plates were incubated at 42°C for 48 h under microaerophilic conditions. Direct bacterial counts were recorded and converted to log CFU per g of fecal samples for statistical analysis. All the birds were individually weighed at the beginning and at the end of the study period (d 42) and feed consumption data were recorded. Cecal samples were collected ($n = 10$ birds/pen), serially diluted and plated on XLD agar for *Salmonella* spp. and on CLA for *Campylobacter* spp. for enumeration as above mentioned. An additional 5 birds per pen were dissected and the contents of crop, gizzard, duodenum, proximal jejunum, proximal ileum, a single ceca, and proximal colon were collected into 2 mL tubes. One chicken from the trans-cinnamaldehyde supplemented diet group did not have any content in the crop. All gastrointestinal content samples collected for phytochemical determination were immediately frozen on dry ice and stored at −80°C until extraction for analysis by GC-MS.

Sample Extraction for Phytochemical Determination

Approximately 200 mg of gastrointestinal content were individually weighed and placed into reinforced 2 mL tubes each containing 6 ceramic beads. One mL of mass spectrometry-grade chloroform was added to the tube and then tubes were placed onto wet ice. Tubes were homogenized using a vortex mixer for 10 min, after which samples were centrifuged at 12,000 × *g* and 4°C for 5 min. Following centrifugation, 600 μL of supernatant was collected, passed through a 0.22 μm syringe filter (for syringe, ThermoFisher Catalog#: S7510-1; for syringe filter, ThermoFisher Catalog#: F2513-4), and filtrate collected into 1.5 mL tubes. Filtrate was then immediately transferred to individual GC vials (VWR Catalog#: 89523-484A), to which 6 μL of an internal standard (octadecanoic methyl ester prepared from trioctadecanoyl glycerol Item No. 23335, CAS No. 555-43-1, Cayman chemicals) was added to each sample. Vials were then immediately stored at −20°C until injection into the GC and analysis by GC-MS. A standard curve was created using the same trans-cinnamaldehyde and caprylic acid that were utilized for the generation of feeds used in the present study (Thermo-Fisher

Scientific Inc., Waltham, MA for trans-cinnamaldehyde, Catalog#: A14689.36; for caprylic acid, Catalog#: 129390010).

Gas Chromatography-Mass Spectrometry

Samples were analyzed using an Agilent 6890N gas chromatograph with 5973 single quadrupole mass spectrometer (**GC/MS**) equipped with an Agilent 7683B autosampler. An Agilent 30m G3903-63011 DB-FastFAME column (internal diameter 0.25 mm and 0.25 μm film liquid layer) was used for the analysis. A sample volume of 1 μL was injected in spitless mode to the injector port where the temperature was maintained at 280°C. Temperature of the oven initially held at 60°C for 3 min followed by temperature ramp from 60°C to 220°C at a rate of 20°C/min followed by a 5 min hold. The carrier gas was helium, the flow rate was variable throughout the run as method used constant pressure, and the mass spectrometer was scanned in the m/z range of 30 to 500 in fast scanning mode. Trans-cinnamaldehyde and caprylic acid identification was performed using both retention time and 70 eV electron impact ionization fragment ion spectra of corresponding analytical standards (Thermo-Fisher Scientific) in NIST search 2.0 library (v2.0). Each compound was quantified by using a calibration curve generated in the concentration range of 0.1 mg/mL (100 ppm) to 0.00001 mg/mL (0.01 ppm) for 5 calibration points. Relative intensities (intensity for phytochemical/intensity of internal standard) instead of absolute intensities for each concentration of phytochemical were used for the calibration curves to correct for injection volume during the sample injection into the GC-MS.

Statistical Analysis

Intestinal content phytochemical data were checked for normal distribution using the D'Agostino & Pearson test, and then analyzed with outliers removed (Grubb's test, alpha $P = 0.05$) using 1-way ANOVA followed by Tukey post hoc test (GraphPad Prism version 10.0.2, La Jolla, CA). Bird bodyweight and log-transformed bacterial count datasets was analyzed using 1-way ANOVA followed by Tukey post hoc test. Differences were considered significant at the threshold of $P < 0.05$.

RESULTS

Bodyweight

Bird bodyweight at the end of the study (d 42 of age) did not significantly differ between the control and caprylic acid groups (Figure 1A). Birds belonging to the trans-cinnamaldehyde group showed a significantly ($P < 0.05$) lesser bodyweight at d 42 of age compared to both control and caprylic acid groups (Figure 1A). Trans-cinnamaldehyde groups showed significant reduction in feed consumption and body weight gain compared to the control or caprylic acid groups (data not shown).

Foodborne Bacterial Colonization

The cloacal swabs from day-of-hatch birds were negative for both *Salmonella* and *Campylobacter*, however fecal samples tested positive for *Salmonella* in all pens at all collection times (data not shown). *Campylobacter* counts were below detection limits in all the treatment groups including control, throughout the study (both in weekly fecal samples and cecal contents collected; data not shown). The 0.75% trans-cinnamaldehyde group showed a significant ($P < 0.05$) reduction in cecal *Salmonella* concentrations when compared to the control, whereas *Salmonella* counts were below detection limits in 0.75% caprylic acid group (Figure 1B).

Trans-Cinnamaldehyde Concentrations in the Chicken Gastrointestinal Tract

Chickens that were provided the trans-cinnamaldehyde supplemented diet had detectable concentrations of trans-cinnamaldehyde in the luminal content of each region of the gastrointestinal tract (Table 1). Concentrations of trans-cinnamaldehyde in the crop and gizzard were significantly ($P < 0.05$) greater than the concentrations detected in the duodenum, jejunum, ileum, ceca, and colon. Crop and gizzard concentrations did not significantly ($P > 0.05$) differ from each other. Duodenal, jejunal, ileal, cecal, and colonic concentrations of trans-cinnamaldehyde were not significantly ($P > 0.05$) different from one another. As expected, trans-cinnamaldehyde was not detected in any region of the gastrointestinal tract of chickens belonging to either the control diet group or the caprylic acid supplemented groups.

Caprylic Acid Concentrations in the Chicken Gastrointestinal Tract

Caprylic acid was detected in crop, gizzard, duodenal, jejunal, and ileal content of chickens fed the caprylic acid supplemented diet (Table 1). Caprylic acid was not detected in ceca or colon content. Crop and gizzard concentrations of caprylic acid were significantly ($P < 0.05$) greater than concentrations found in the duodenum, jejunum, and ileum. Crop and gizzard concentrations were not significantly ($P > 0.05$) different from one another. As expected, caprylic acid was not detected in the content of any gastrointestinal region of chickens from either the control or trans-cinnamaldehyde supplemented group.

DISCUSSION

Global demand for poultry products is only expected to increase despite already being the most consumed animal protein in the world (Miller et al., 2022). As such,

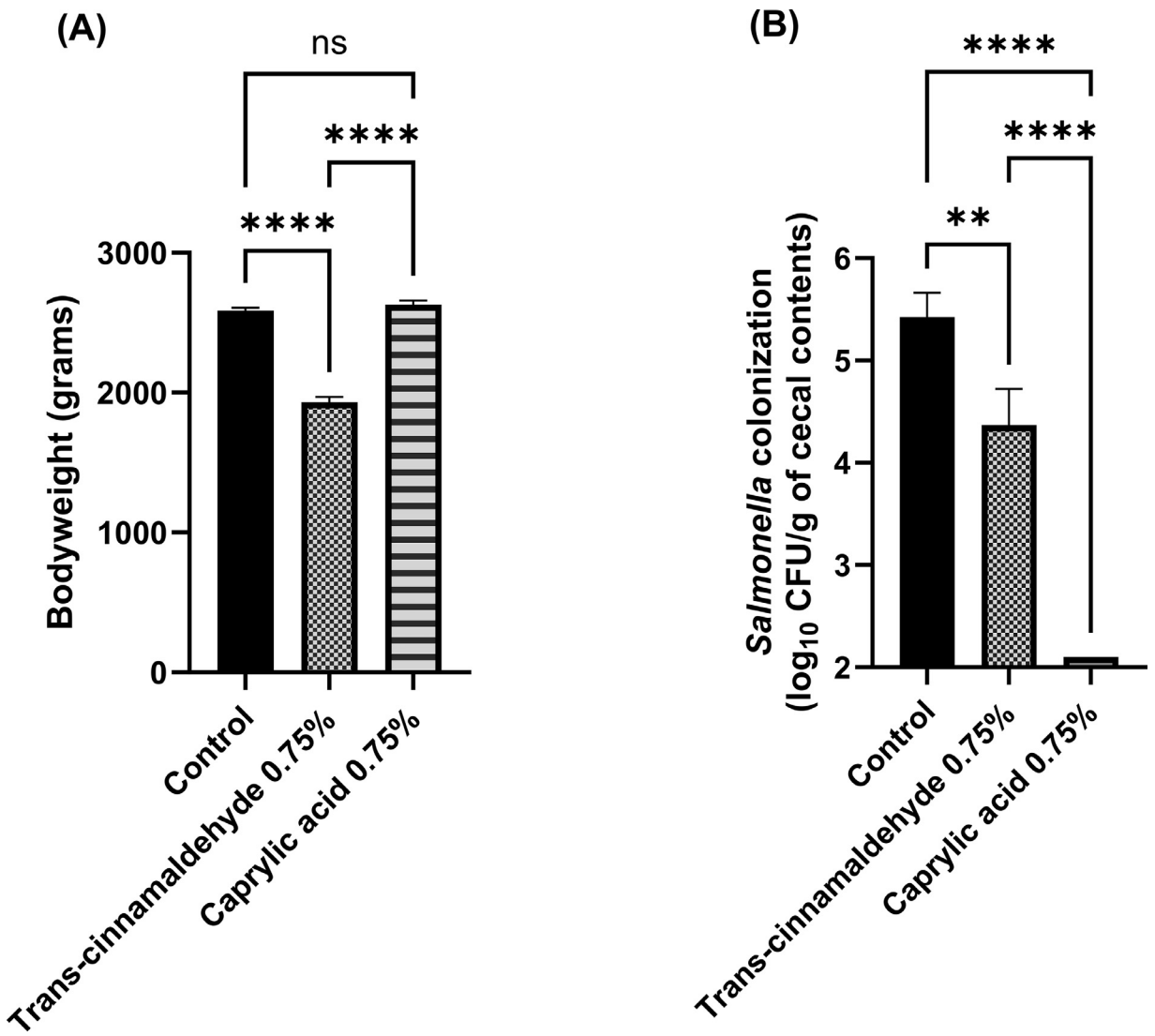

Figure 1. The impact of feed-supplemented phytochemicals on broiler chicken bodyweight and cecal colonization by *Salmonella* spp. (A) Bodyweights (grams) of broiler chickens at d 42 of age provided either control (corn/soy diet), 0.75% trans-cinnamaldehyde supplemented diet, or 0.75% caprylic acid supplemented diet. Each bar includes 2 replicates (36−40 chickens per replicate). (B) *Salmonella* spp. log-transformed counts obtained from d 42 of age broiler chicken cecal contents ($\log_{10}$ CFU/g of cecal content). Each bar includes 2 replicates (10 chickens per replicate). All values are expressed as mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$; ns = not statistically significant ($P > 0.05$). Data were analyzed using 1-way ANOVA followed by Tukey's post hoc test as described in the Materials and Methods section.

there exists an urgent need to address poultry carriage of bacterial pathogens that are a major cause of human foodborne illness worldwide. A variety of plant-derived compounds exert antimicrobial activity and have been demonstrated to reduce carriage of foodborne pathogens in poultry. Yet, in poultry, the fate of these phytochemicals in the gastrointestinal tract after being consumed in-feed is unknown. Major poultry-related foodborne

Table 1. Phytochemical concentrations in the luminal content of each region of the broiler chicken gastrointestinal tract.

	Control diet		TC supplemented diet		CA supplemented diet	
Gastrointestinal tract region	TC	CA	TC	CA	TC	CA
Crop	ND	ND	56.20 ± 7.71^{a}	ND	ND	123.00 ± 20.33^{a}
Gizzard	ND	ND	56.96 ± 6.82^{a}	ND	ND	115.50 ± 18.12^{a}
Duodenum	ND	ND	0.15 ± 0.02^{b}	ND	ND	8.21 ± 1.72^{b}
Jejunum	ND	ND	1.83 ± 0.18^{b}	ND	ND	1.55 ± 0.67^{b}
Ileum	ND	ND	2.96 ± 0.18^{b}	ND	ND	0.24 ± 0.14^{b}
Ceca	ND	ND	0.73 ± 0.12^{b}	ND	ND	ND
Colon	ND	ND	2.78 ± 0.17^{b}	ND	ND	ND

Values with different superscript letters within columns denote significant difference ($P < 0.05$) compared between gut regions within the same dietary group. Control (corn/soy feed), TC = trans-cinnamaldehyde 0.75% supplemented in feed, CA = caprylic acid 0.75% supplemented in feed, ND = not detectable. Values are ng of phytochemical per mg of gut luminal content. All values are expressed as mean ± SEM ($n = 10$ chickens/diet group). Data were analyzed with outliers removed (Grubb's test) using 1-way ANOVA followed by Tukey's post hoc test as described in the Materials and Methods section.

pathogens, including *Campylobacter jejuni* and *Salmonella* spp. are often found in highest abundances in regions of the lower gastrointestinal tract, thereby underscoring the need to understand if in-feed provision of phytochemicals results in their delivery to relevant physiological sites in the chicken gut. As such, the objective of the present study was to determine whether 2 well-studied phytochemicals, trans-cinnamaldehyde and caprylic acid, when provided in-feed reach each region of the broiler chicken gastrointestinal tract and reduce carriage of naturally colonized bacterial foodborne pathogens.

In the present study, trans-cinnamaldehyde and caprylic acid supplementation in-feed were found to significantly reduce *Salmonella* spp. carriage in the broiler chicken gut. These findings are in agreement with past reports which utilized a similar inclusion level in broiler feed for both trans-cinnamaldehyde (Kollanoor-Johny et al., 2012a) and caprylic acid (Johny et al., 2009; Kollanoor-Johny et al., 2012b). While past studies have utilized pathogen challenge models in assessing phytochemical efficacy, we sought to evaluate how phytochemical inclusion in feed may prevent enteric colonization with *Salmonella* and *Campylobacter* populations acquired naturally from the production environment. As such, birds were provided phytochemical supplemented feeds immediately posthatch and continuing through the end of the study at d 42 of age. While cloacal swabs confirmed each group to be *Salmonella* and *Campylobacter* free at placement, provision of phytochemical supplemented feeds appears to have had a protective effect against environmental acquisition of *Salmonella*. Indeed, a previous study demonstrated that caprylic acid included in broiler feed as a prophylactic reduced cecal carriage of *Salmonella* Enteritidis following challenge with this bacterial organism (Johny et al., 2009). Caprylic acid supplementation showed similar weight gain and feed efficiency compared to the conventional control diet, however, in-feed supplementation of trans-cinnamaldehyde (0.75% in feed) significantly reduced the feed intake and body weight gain.

Feed supplemented with caprylic acid was found to exert a more profound effect on broiler cecal carriage of *Salmonella* compared to feed that contained trans-cinnamaldehyde. Interestingly, trans-cinnamaldehyde, but not caprylic acid, was detected in cecal contents thereby suggesting the site of *Salmonella* inhibition by caprylic acid may be higher in the broiler gastrointestinal tract. Indeed, it is important to acknowledge it was previously hypothesized that the site of action of caprylic acid on bacterial foodborne pathogens is the broiler crop and stomach (Hermans et al., 2012). As *Salmonella* is typically acquired via the oral route, it is reasonable to assume contact between consumed diet and bacterial cells in the upper gastrointestinal tract. Contact with medium chain fatty acids was previously demonstrated to have a direct effect in reducing *Salmonella* spp. invasive capability (Van Immerseel et al., 2004). Moreover, a previous study reported that broiler chickens which received caprylic acid supplemented in-feed exhibited reduced colonization in ceca by *C. jejuni* following challenge without altering cecal microbial populations (Solís de los Santos et al., 2010). As medium chain fatty acids have been demonstrated to affect a wide range of bacteria, including nonpathogens, commonly found in the lower gut of both avian (Chen et al., 2020) and mammalian (Roopashree et al., 2021) species, this likely suggests that the inclusion level needed to protect broiler chickens against foodborne pathogens is low enough to be completely absorbed before reaching the ceca. Future studies are also warranted to investigate if secondary compounds that may result from host or microbial metabolism of caprylic acid may also exert antimicrobial activity and might reach other gastrointestinal regions.

Conversely, trans-cinnamaldehyde was detected in each region of the broiler gastrointestinal tract. Although previous studies have found trans-cinnamaldehyde to reduce cecal populations of *Salmonella* and *Campylobacter*, the inhibitory effect of trans-cinnamaldehyde may be dependent on the specific bacterial strains (Hermans et al., 2011). Also, it is possible that reducing the level of inclusion or encapsulation to mask the flavor may provide the desired reduction in pathogen colonization without affecting the production performance parameters. The present findings on the in vivo distribution of caprylic acid and trans-cinnamaldehyde could be used to cooperatively target foodborne bacterial pathogens that colonize the chicken gastrointestinal tract. Indeed, as trans-cinnamaldehyde reached the ceca, this could help address previous reports where cecal mucus was found to protect *C. jejuni* from the bactericidal effects of caprylic acid in chickens (Hermans et al., 2010).

Together, our findings demonstrate that in-feed supplementation of trans-cinnamaldehyde and caprylic acid, 2 well-studied phytochemicals, exhibit different spatial distribution in the broiler chicken gut while exerting clear protective effects against cecal carriage of environmentally acquired *Salmonella*. Further studies are warranted to evaluate feed supplementation formulations that utilize both caprylic acid and trans-cinnamaldehyde.

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DISCLOSURES

The authors declare no conflicts of interest.

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