Research Note: Virulence gene downregulation and reduced intestinal colonization of *Salmonella enterica* serovar Typhimurium PHL2020 isolate in broilers by a natural antimicrobial (NeutraPath™)

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ABSTRACT The reduction in antibiotic growth promoter use in poultry, due to antibiotic resistance concerns, has created a need for natural solutions that control enteric pathogens like *Salmonella*. One of these natural feed additives, a select blend of essential oils, fatty acids, and an enterosorbent mineral (NeutraPath), was assessed for its effects on the intestinal colonization of *Salmonella enterica* serovar Typhimurium PHL2020 isolate (ST-PHL2020) in broiler chickens and ST-PHL2020 virulence gene expression. An in vitro digestion model simulating the pH and enzymatic conditions of 3 gastrointestinal compartments (crop, proventriculus, and intestine) was first used to evaluate the antibacterial effects of NeutraPath on ST-PHL2020. For the in vivo study, day-old male broilers (n = 90) were randomly allocated to 1 of 3 groups: control or NeutraPath supplemented at 0.25 or 0.5%. The dose rates were chosen to enable observable statistical effects during high *Salmonella* challenge. All groups were challenged with ST-PHL2020 (10^6 cfu/bird) via oral gavage on day 9. Bacterial load and prevalence of ST-PHL2020 were examined in ceca-cecal tonsils, and intestinal permeability was assessed via serum recovery of fluorescein isothiocyanate dextran (**FITC-d**) 24 h postchallenge. NeutraPath inhibited ($P < 0.05$) ST-PHL2020 growth in the in vitro digestion model compared to the control at all concentrations and in all compartments other than NeutraPath 0.25% in the crop. In vivo, NeutraPath 0.25 and 0.5% reduced ($P < 0.05$) the total cfu recovered and total prevalence of ST-PHL2020 in the ceca. The serum FITC-d levels were also reduced ($P < 0.05$) by NeutraPath. Further, NeutraPath's effects on ST-PHL2020's *Salmonella* pathogenicity island-1 virulence network development were explored via treating ST-PHL2020 at subinhibitory concentration (1 mg/mL) of NeutraPath in vitro. Compared to the control, NeutraPath downregulated ST-PHL2020 *hilA* and *invF* mRNA expression, which further blocked expression of key downstream effectors involved in ST-PHL2020 invasion. Collectively, NeutraPath has the potential to reduce ST-PHL2020 intestinal colonization in broilers and preserve intestinal barrier integrity.

Key words: *Salmonella* Typhimurium PHL2020 isolate, intestinal colonization, NeutraPath, virulence gene expression, natural antimicrobial

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INTRODUCTION

Salmonella is one of the most common food-borne pathogens throughout the world, with poultry products a frequent source of contamination (Cosby et al., 2015). To enter the food chain, *Salmonella* first colonizes the birds' intestinal tract before being passed to humans through contamination of meat and other poultry products (Cosby et al., 2015). Thus, on-farm strategies to reduce or eliminate *Salmonella* from the source are fundamental and key to successful control of S*almonella* contamination of the whole food chain.

Historically, subtherapeutic levels of antibiotics (antibiotic growth promoters) fed to poultry to improve production efficiency could also control enteric pathogens such as *Salmonella* species. However, concern over the increase in antimicrobial resistant pathogens has led to restrictions in the use of antibiotic growth promoters. Therefore, innovative antibiotic-free alternatives that can reduce the incidence of enteric pathogens in poultry at the on-farm level are warranted.

Specific natural ingredients have been demonstrated to possess potent bactericidal or bacteriostatic efficacy toward *Salmonella* species (Ebani et al., 2019). NeutraPath, available for sale in select international

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geographies, is a formulated natural feed additive that contains a select blend of essential oils, fatty acids and a thermally processed enterosorbent mineral. In previous broiler chicken research, NeutraPath reduced the prevalence of *Salmonella enterica* serovar Heidelberg in cecal contents and the number of *Salmonella* Heidelberg positive cloacal swabs (Xue et al., 2018).

Apart from colonization in the gut lumen, *Salmonella* species are equipped with sophisticated virulence machinery enabling them to interplay with intestinal epithelial cells and mount infection (Lou et al., 2019). The syringe-like type III secretion system 1 (**TTSS-1**), encoded by *Salmonella* pathogenicity island-1 (**SPI-1**), is essential for *Salmonella* species to invade the host's non-phagocytic intestinal epithelial cells and proceed with other intracellular phenotypes (intracellular survival and replication) (Lou et al., 2019).

The current study aimed to investigate the antimicrobial effects of NeutraPath on *Salmonella enterica* serovar Typhimurium strain PHL2020 (**ST-PHL2020**) by using an in vitro simulated digestion model and an in vivo study encompassing cecal colonization and intestinal integrity. Furthermore, NeutraPath's effects on ST-PHL2020's SPI-1 virulence network development were explored via treating ST-PHL2020 with a subinhibitory concentration of NeutraPath in vitro.

MATERIALS AND METHODS

Salmonella *Typhimurium Strain*

A poultry isolate of *Salmonella enterica* serovar Typhimurium PHL2020 strain was obtained from the USDA National Veterinary Services Laboratory (Ames, IA) and used in all experiments. The isolate was resistant to 25 μg/mL of novobiocin (**NO**, catalog no. N1628, Sigma-Aldrich, MilliporeSigma, St. Louis, MO) and selected for resistance to 20 μg/mL of nalidixic acid (**NA**, catalog no. N4382, Sigma-Aldrich) in our laboratory.

Determination of Minimum Inhibitory Concentration of NeutraPath

In vitro assays were performed to determine the minimal inhibitory concentration (**MIC**) of NeutraPath. For the MIC assay, ST-PHL2020 was inoculated in a lysogeny broth medium (catalog no. 12780029, Thermo Fisher Scientific, Waltham, MA) in the presence of different concentrations (0, 1, 3, and 5 mg/mL) of NeutraPath. The MIC value was determined after incubation for 16 h at 37°C.

In Vitro Digestion Model

An in vitro digestion model was used to evaluate the ability of NeutraPath to neutralize ST-PHL2020 in a simulated gastrointestinal environment. The basal diet and 3 treatments supplemented with 0.25, 1, and 5% NeutraPath were assessed. The model consisted of 3 gastrointestinal compartments simulated to match the pH and enzymes present in broilers. Briefly, quintuplicates of each treatment were inoculated with 10^8 cfu/tube of ST-PHL2020 and maintained at 40°C to simulate poultry body temperature. The first gastrointestinal compartment simulated was the crop, where 5 g of feed and 10 mL of 0.03 M hydrochloric acid (catalog no. HX0607-2, Supelco, MilliporeSigma) were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously, reaching an approximate pH of 5.20, then incubated for 30 min. The second gastrointestinal compartment simulated was the proventriculus, where 3,000 units of pepsin per g of feed (catalog no. P7000, Sigma-Aldrich) and 2.5 mL of 1.5 M hydrochloric acid were added to each of the tubes, reaching a pH between 1.4 and 2.0, then incubated for 45 min. The final gastrointestinal compartment simulated was the intestinal section. For this compartment, 6.84 mg of 8 × pancreatin (catalog no. P7545, Sigma-Aldrich) was used per g of feed and included in 6.5 mL of 1.0 M sodium bicarbonate (catalog no. S6014, Sigma-Aldrich). The pH ranged between 6.4 and 6.8 and all tubes were incubated for 2 h. After the incubation time in each compartment, a sample was collected to enumerate ST-PHL2020 as described in the following *Salmonella* Recovery and Enumeration section.

In Vivo Study Diet Preparation

The basal experimental diet was a commercial broiler diet formulated to approximate the nutritional requirements of broiler chickens as recommended by the NRC. No antibiotics were added to the diet. NeutraPath (Amlan International, Chicago, IL) contains a proprietary blend of essential oils, medium chain fatty acids and an enterosorbent mineral composed primarily of calcium montmorillonite and opal-CT lepispheres. NeutraPath is a commercial product sold in various international markets outside the U.S.

In Vivo Experimental Design

All animal handling procedures complied with the Institutional Animal Care and Use Committee at the University of Arkansas, Fayetteville, AR (protocol no. 15006). Day-old male Cobb-Vantress broiler chicks (Fayetteville, AR) were randomly allocated to 1 of 3 groups (n = 30 broilers): challenged control fed the basal diet and 2 groups supplemented with either 0.25 or 0.5% NeutraPath. The NeutraPath dose rates used in this proof of concept study were chosen to enable statistical effects to be observed during experimentally induced high *Salmonella* challenge. Recommended commercial feed rates of NeutraPath are typically 0.1%, corresponding to the lower pathogen levels associated with naturally occurring *Salmonella* challenge. Broilers were housed in heated brooder batteries and kept in controlled age-appropriate environmental conditions with ad libitum access to feed and water.

Upon arrival, the ceca-cecal tonsils, liver and spleen were aseptically cultured in tetrathionate enrichment broth (catalog no. 210420, BD Difco, Sparks, MD). Enriched samples were confirmed negative for *Salmonella* by streak plating the samples on xylose lysine tergitol-4 (catalog no. 223410, BD Difco) selective media. At 9-day-old, all broilers were orally gavaged with 10^6 cfu of live ST-PHL2020 per chicken. At 24 h postchallenge, chickens in all groups were given an oral gavage dose of fluorescein isothiocyanate dextran (**FITC-d**, 8.32 mg/kg) 1 h before the chickens were euthanized by CO_2 inhalation. Blood samples were collected from the femoral vein for serum FITC-d determination. Intestinal leakage of FITC-d was used as a marker of paracellular transport and mucosal barrier dysfunction by measuring its serum concentration. Ceca-cecal tonsils were removed from 12 birds per group to evaluate *Salmonella* recovery. Ceca-cecal tonsils were homogenized and diluted with saline (1:4 by wt/vol) and tenfold dilutions were plated on brilliant green agar with NO and NA, incubated at 37°C for 24 h to enumerate total ST-PHL2020 cfu. Subsequently, the ceca-cecal tonsil samples were enriched in 2 × concentrated tetrathionate enrichment broth and further incubated at 37°C for 24 h. Enrichment samples were streaked onto xylose lysine tergitol-4 with NO and NA selective media for confirmation of *Salmonella* presence.

Virulence Gene Expression

ST-PHL2020 was grown with aeration at 37°C in LB medium (catalog no. 12780029, Thermo Fisher Scientific) supplemented with or without 1 mg/mL of NeutraPath for 12 h to an OD600 of 1.3. Cells were harvested by centrifugation for 5 min at 8,000 × *g* and RNA was extracted using the RNeasy Protect Bacteria Mini Kit (catalog no. 74524, Qiagen, Hilden, Germany). RNA was reverse-transcribed using random primers (catalog no. 48190011, Invitrogen by Thermo Fisher Scientific) and Superscript III Reverse Transcriptase (catalog no. 18080093, Invitrogen by Thermo Fisher Scientific) at 50°C for 1 h. QuantStudio 5 Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific) was used to perform qPCR experiment. Primers were designed using the Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/) and reference genes from the NCBI database (https://www.ncbi.nlm.nih.gov/): *hilA* (Gene ID: 1254399) (CAGGGCTATCGGTTTAATCGT; ACTGCGATAATCCCTTCACG), *sopB* (Gene ID: 1252609) (TATGAGGGAAAGGGCGTATG; TTCATGATAGGGGGAAAGCA), *sopE* (GenBank ID: AAC02071.1) (TCGGCAGTTTACAGCAAGAA; GCTTGGCGTAAAAACGTCAT), *avrA* (Gene ID: 1254388) (GAAACCGATCTCGAAATGA; GAGAAATCGGGCAGATTCAA), *rpoD* (Gene ID: 1254734) (GCTGCTGCACAAGTTCTGTC; GGTTGATCCCGTCTTCGATA), *16S rDNA* (Gene ID: NR_074910) (GTATGCGCCATTGTAGCACG; TCATCATGGCCCTTACGACC), *sipA* (GeneID:1254405) (GCCAGCTGCATTGAACATAA; ACGCTGGTACCCTGCTCTT) and *invF* (Gene ID: 1254422) (CCAGCATTCTCATCGTGTTG; AACGCTGCGAGTACTGGTTT). Aliquots (1 μL) of cDNA (1 ng per reaction) were used as template for qPCR with SYBR Green PCR Master Mix (catalog no. 4309155, Applied Biosystems) and primers (500 nM final concentration). Determined threshold cycle (**CT**) values were then used for further $\Delta\Delta$CT analysis. The 16S rDNA and *rpoD* genes were used as the reference genes to normalize samples, and similar results were obtained. A relative quantification value was calculated for each gene with the control group as a reference.

Statistical Analysis

Samples per variable in the in vitro digestion ST-PHL2020 enumeration (n = 5); in vivo ST-PHL2020 cecal colonization data (n = 12); and serum FITC-d concentration (n = 30), implying a normal distribution (Shapiro-Wilk test), and the homoscedasticity was verified (Levene's test). Accordingly, the parametric test of analysis of variance as a completely randomized design using the GLM procedure of SAS was performed. The differences between the means were evaluated using Duncan's honestly significant difference (**HSD**) test, and the P value was established with an alpha level of $P < 0.05$. The cecal prevalence data is expressed as positive/total chickens (%) and was compared using the chi-squared test of independence, testing all possible combinations to determine the significance ($P < 0.05$).

RESULTS AND DISCUSSION

Salmonella remains one of the most common causes of food-borne disease throughout the world. Along with its widespread prevalence, another concern is the discovery of antimicrobial-resistant strains of *Salmonella*. The rise in antimicrobial-resistant pathogens has contributed to restrictions in the use of antibiotic growth promoters and created the need for alternative antibiotic-free pathogen control methods. In the current study, we generated multiple lines of evidence demonstrating that a select blend of essential oils, fatty acids and a thermally processed enterosorbent mineral (NeutraPath) exhibits a potent antimicrobial effect against *S.* Typhimurium strain PHL2020 and can reduce its intestinal colonization.

In the present study, the MIC and subinhibitory concentration (**SIC**) of NeutraPath against ST-PHL2020 was first determined to be 5 mg/mL and 0.5 mg/mL, respectively. We further demonstrated that NeutraPath administered at the comparable concentrations to its MIC can inhibit ST-PHL2020 growth in various upper gastrointestinal (**GI**) compartments (i.e., crop, proventriculus, and upper small intestine) by using an in vitro system mimicking the in vivo digestion physiology as presented in Table 1. The lowest concentration of NeutraPath (0.25%) reduced ($P < 0.05$) ST-PHL2020

Table 1. NeutraPath inhibition of *Salmonella enterica* serovar Typhimurium strain PHL2020 colonization in the crop, proventriculus, and intestine compartments of an in vitro digestion model.

	S. Typhimurium (Log_{10} cfu/mL)		
Treatments	Crop	Proventriculus	Intestine
Control	7.62 ± 0.15^{a}	4.34 ± 0.03^{a}	5.04 ± 0.04^{a}
NeutraPath 0.25%	7.42 ± 0.05^{ab}	4.04 ± 0.0^{b}	4.24 ± 0.06^{b}
NeutraPath 1%	7.24 ± 0.06^{b}	0.00 ± 0.0^{c}	3.24 ± 0.06^{c}
NeutraPath 5%	5.04 ± 0.04^{c}	0.00 ± 0.0^{c}	0.0 ± 0.0^{d}

Ten tubes were inoculated with 10^8 *S.* Typhimurium in each treatment for each compartment. Data are expressed as mean Log_{10} cfu/mL ± standard error (n = 10).

[a-d]Means within a column lacking a common superscript differ ($P < 0.05$).

colonization in the proventriculus and intestine compartments compared to the control. This inhibition of ST-PHL2020 growth increased as NeutraPath concentration increased. The 1% NeutraPath treatment reduced ($P < 0.05$) ST-PHL2020 colonization in the crop and intestine compared to the control, and no ST-PHL2020 cfu were detected in the proventriculus. The 5% NeutraPath treatment also reduced ($P < 0.05$) ST-PHL2020 colonization in the crop compartment, and there were no detectable ST-PHL2020 cfu in the proventriculus or intestine. Further, we proved that NeutraPath at 0.25 and 0.5% reduced ST-PHL2020 colonization of the ceca-cecal tonsils evidenced by both the bacterial load (total cfu recovered) and total prevalence of ST-PHL2020 in the ceca being significantly reduced compared to the control ($P < 0.05$, Table 2). Our findings are consistent with previous research showing specific essential oils and medium chain fatty acids (Ebani et al., 2019) had direct bactericidal or bacteriostatic effects against *Salmonella* spp. in vitro and decreased *Salmonella* spp. populations in chicken ceca in vivo (Kollanoor-Johny et al., 2012).

In order to survive in the host, *Salmonella enterica* have evolved an intricate and sophisticated virulence network to manipulate multiple aspects of host defense mechanisms, and cell physiology and signal transduction to promote their replication in the host (Lou et al., 2019). The ability of NeutraPath to modulate ST-PHL2020 virulence network development was also demonstrated in the current study. When treated with NeutraPath at the subinhibitory concentration (0.5 mg/mL), which was 10 times lower than the MIC, ST-PHL2020 *hilA* and *invF* mRNA expression was downregulated 1.71- and 10.71-fold, respectively compared to the control. Further, the *sopB, sopE, sipA*, and *avrA* mRNA expression was downregulated 3.22-, 1.74-, 1.09-, and 1.68-fold, respectively compared to the control. The *hilA* and *invF* genes are located within and regulate the SPI-1 that encodes the proteins necessary for TTSS-1 assembly (Lou et al., 2019). The TTSS-1, an important virulence factor, delivers effector proteins to the host cell via a needle complex (Lou et al., 2019). This system is often what determines the level of pathogenesis, as the expression levels of SPI-1 genes are correlated to the ability of *Salmonella* to invade intestinal cells (Lou et al., 2019). Suppression of the HilA-InvF axis by NeutraPath at this subinhibitory concentration further blocked expression of key downstream effectors involved in Salmonella invasion (*sopB, sopE*, and *sipA*). The effectors *sopB* and *sopE* stimulate inflammation and assist invasion, whereas *sipA* promotes the efficiency of host cell invasion (Lou et al., 2019). Particularly, these 3 effectors are also involved in disrupting intestinal epithelial cell tight junction structure and function and help *Salmonella* species penetrate the epithelial barrier, which is kept closed by tight junctions (Boyle et al., 2006). These *Salmonella* SPI-1 effectors (*sopB, sopE*, and *sipA*) can dramatically change the localization, expression, and phosphorylation of key tight junction proteins such as ZO-1 and occludin (Boyle et al., 2006), which, consequently, leads to increased gut permeability. In this study, NeutraPath treatment strikingly downregulated the expression of these SPI-1 effectors and conferred preservation of the functional integrity of gut barrier after ST-PHL2020 challenge evidenced by the significantly reduced presence of FITC-d in serum (vs. control, $P < 0.05$, Table 2).

The cecal colonization and prevalence of ST-PHL2020 was reduced by NeutraPath in the current study and was likely due to a combination of bactericidal or bacteriostatic effects in the upper GI tract and antivirulence effects in the low GI tract. When first ingested, supplementation of NeutraPath at 0.25 or 0.5% is likely to result in a concentration of NeutraPath's antimicrobial constituents (i.e., medium chain fatty acids and essential oil compounds) comparable to the MIC level in the initial GI segments (i.e., crop, proventriculus, and proximal small intestine, Zentek et al., 2012). The in vitro digestion model further confirmed that NeutraPath can directly reduce the bacterial load of ST-PHL2020 in these segments. However, these compounds are subjected to extensive absorption in the cranial parts of the digestive tract (Zentek et al., 2012, Oceľová et al., 2019). Previous research on the GI passage kinetics of these compounds demonstrates that their concentration in the

Table 2. NeutraPath inhibition of *Salmonella enterica* serovar Typhimurium strain PHL2020 (ST-PHL2020) colonization in the ceca-cecal tonsils of broiler chickens and reduced in vivo intestinal permeability after *Salmonella* challenge.

Treatment	*S.* Typhimurium Cecal Colonization[1]	*S.* Typhimurium Prevalence[2]	Serum Fluorescein Isothiocyanate Dextran (FITC-d, ng/mL)
Control	3.28 ± 0.22^{a}	$12/12\ (100.00\%)^{a}$	263.44 ± 21.32^{a}
NeutraPath 0.25%	1.44 ± 0.39^{b}	$7/12\ (58.33\%)^{b}$	123.65 ± 25.00^{b}
NeutraPath 0.5%	1.49 ± 0.33^{b}	$8/12\ (66.66\%)^{b}$	150.53 ± 22.60^{b}

[a-b]Means within a column lacking a common superscript differ ($P < 0.05$).

[1]Data are expressed as mean Log_{10} cfu/g of tissue from 12 birds/group ± standard error.

[2]Data are expressed as *S.* Typhimurium positive/12 birds per group (%).

ileal or cecal content can be reduced by 10 to 100 folds compared to that of the stomach digesta (Zentek et al., 2012, Oceľová et al., 2019). Thus, when traveling down the GI tract, these antimicrobial constituents in NeutraPath formula are likely to be in the SIC range in the lower GI tract, the primary colonization site of *Salmonella*. Although these antimicrobial compounds at SIC levels do not kill bacteria, they can change the cell surface physico-chemical properties of bacteria and interfere with some important bacterial behaviors involved in bacterial virulence (Braga et al., 2000). As evidenced in the current study, NeutraPath at the SIC level did exhibit antivirulence properties via blocking the SPI-1 TTSS-1 machinery of ST-PHL2020.

Collectively, NeutraPath had the potential to reduce ST-PHL2020 intestinal colonization in broilers and preserve the functional integrity of the intestinal barrier during ST-PHL2020 challenge. In addition to direct bacteriocidal or bacteriostatic effects, this formula also exhibits antivirulence properties by downregulating ST-PHL2020's HilA-InvF axis and its target SPI-1 effector protein gene expression. Nevertheless, the antimicrobial susceptibility and resistance profiles could vary widely between *S.* Typhimurium strains and there is marked strain heterogeneity in SPI-1 expression, regulation, and invasive phenotype amongst different *S.* Typhimurium strains (Clark et al., 2011). Further investigation is still needed to evaluate whether the antimicrobial and antivirulence properties of NeutraPath are more broadly applicable to other *S.* Typhimurium isolates from different host and sources.

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DISCLOSURES

H. X. and D. W. are employees of Amlan International. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships construed as a potential conflict of interest.

REFERENCES

Boyle, E. C., N. F. Brown, and B. B. Finlay. 2006. *Salmonella enterica* serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function. Cell. Microbiol. 8:1946–1957.

Braga, P. C., M. D. Sasso, and M. T. Sala. 2000. Sub-MIC concentrations of cefodizime interfere with various factors affecting bacterial virulence. J. Antimicrob. Chemother. 45:15–25.

Clark, L., C. A. Perrett, L. Malt, C. Harward, S. Humphrey, K. A. Jepson, I. Martinez-Argudo, L. J. Carney, R. M. La Ragione, T. J. Humphrey, and M. A. Jepson. 2011. Differences in *Salmonella enterica* serovar Typhimurium strain invasiveness are associated with heterogeneity in SPI-1 gene expression. Microbiology 157:2072–2083.

Cosby, D. E., N. A. Cox, M. A. Harrison, J. L. Wilson, R. J. Buhr, and P. J. Fedorka-Cray. 2015. *Salmonella* and antimicrobial resistance in broilers: a review. J. Appl. Poult. Res. 24:408–426.

Ebani, V. V., S. Nardoni, F. Bertelloni, G. Tosi, P. Massi, L. Pistelli, and F. Mancianti. 2019. In vitro antimicrobial activity of essential oils against *Salmonella enterica* serotypes Enteritidis and Typhimurium strains isolated from poultry. Molecules 24:900.

Kollanoor-Johny, A., T. Mattson, S. A. Baskaran, M. A. Amalaradjou, S. Babapoor, B. March, S. Valipe, M. Darre, T. Hoagland, D. Schreiber, M. I. Khan, A. Donoghue, D. Donoghue, and K. Venkitanarayanan. 2012. Reduction of *Salmonella* enterica serovar enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Appl. Environ. Microbiol. 78:2981–2987.

Lou, L., P. Zhang, R. Piao, and Y. Wang. 2019. *Salmonella* pathogenicity island 1 (SPI-1) and its complex regulatory network. Front. Cell. Infect. Microbiol. 9:270.

Oceľová, V., R. Chizzola, G. Battelli, J. Pisarcikova, S. Faix, F. Gai, and I. Placha. 2019. Thymol in the intestinal tract of broiler chickens after sustained administration of thyme essential oil in feed. J. Anim. Physiol. Anim. Nutr. (Berl) 103:204–209.

Xue, H., D. Wang, S. Ching, and S. Johnston. 2018. In vitro and in vivo evaluation of potential antimicrobial effects of NeutraPath™ against *Salmonella* Heidelberg. Poult. Sci. 97(E-Suppl. 2):27 (Abstr).

Zentek, J., S. Buchheit-Renko, K. Männer, R. Pieper, and W. Vahjen. 2012. Intestinal concentrations of free and encapsulated dietary medium-chain fatty acids and effects on gastric microbial ecology and bacterial metabolic products in the digestive tract of piglets. Arch. Anim. Nutr. 66:14–26.