Archives of Animal and Poultry Sciences

Juniper Publishers key to the Researchers

Research Article
Volume 3 Issue 1- February 2025
DOI: 10.19080/AAPS.2025.03.555603

Arch Anim Poult Sci

Beta-Resorcylic Acid and Chitosan Reduce *Salmonella* in Broilers

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Submission: January 27, 2025; **Published:** February 18, 2025

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Abstract

Two generally recognized as safe compounds, β-resorcylic acid (BR, 0.5%, 1%) and chitosan (CH, 0.5%, 1%), along with their combinations (BR 1% + CH 1%; BR 0.5% + CH 0.5%), were evaluated for reducing *Salmonella* Enteritidis (SE) colonization in broiler chickens. One hundred sixty-day-old chicks were divided into eight groups (20 birds/group): (1) negative control (no SE or supplements), (2) CH control, (3) BR control, (4) CH + BR control, (5) positive control (SE challenge only), (6) CH treatment, (7) BR treatment, and (8) CH + BR treatment. CH and BR were fed for 20 days. On day 8, birds in treatments 5-8 were challenged with SE (8 log10 CFU/bird). Thirteen days post-challenge, SE was enumerated in the cecum, liver, and crop. CH (1%) reduced cecal SE by ~2 log10 CFU/g, while BR (1%) reduced SE by ~3 log10 CFU/g. The combination (BR + CH at 0.5% or 1%) achieved reductions of ~2-2.5 log10 CFU/g. In the liver, BR (1%) and CH (1%) reduced SE by ~2.5 and ~1.5 log10 CFU/g, respectively, with their combination reducing SE by ~1.5 log10 CFU/g. CH (1%) and BR (1%) reduced crop SE by ~2.3 and ~1.3 log10 CFU/g, respectively. Treatments at 0.05% did not affect body weight ($P > 0.05$), but 1% CH or BR slightly decreased body weight ($P < 0.05$)

Keywords: Broilers; *Salmonella*; β-Resorcylic acid; Chitosan; Antimicrobial

Abbreviation: CVMDL: Connecticut Veterinary Medical Diagnostic Laboratory; IACUC: Institutional Animal Care and Use Committee

Introduction

Salmonella is the most common food borne pathogen causing 1.35 million illnesses, 26, 500 hospitalizations and 420 deaths in the United States, annually [1]. *Salmonella enterica serovar* Enteritidis (SE) is one of the most common serotypes of *Salmonella* reported worldwide [2]. The total annual cost associated with *Salmonella* in the United States is estimated to be $3.7 billion in which contaminated poultry carcass and eggs constitute the majority [3]. The Centres for Disease Control and Prevention, Atlanta, Georgia reported that despite extensive control efforts, the incidence of infections caused by *Salmonella* has not significantly changed in recent years [1]. SE colonizes various parts of the chicken intestinal tract; cecum being the most common site [4,5]. Cecal colonization of the pathogen eventually leads to fecal shedding, contamination of eggshells with infected feces, carcass contamination during slaughter, and possible retro contamination of reproductive organs [5,6]. Human salmonellosis is caused primarily by the consumption of raw or undercooked chicken meat and eggs, or contaminated products [7,8]. Therefore, reducing the populations of SE in the chicken intestinal tract would potentially decrease contamination of poultry meat and eggs. Furthermore, *Salmonella* control strategies implemented at pre-harvest could lead to microbiologically safer poultry products to the consumer. Many interventions have been studied to control SE at the farm level with varying degrees of success. These include feeding chickens with competitive exclusion bacteria [9,10], bacteriophage [11], enzymes such as xylanase [12], organic acids [13,14], fructooligosaccharides [15], mannanoligosaccharides [12,16], chicory fructans [17], vaccines [18,19] and antibacterial agents furazolidone and furaltadone [20]. Historically, natural and organic compounds have served as a useful resource for the development of novel drugs against human and animal diseases. In recent years, the use of natural compounds has gained attention due to increasing concerns over the safety of synthetic chemicals [21] and emerging antibiotic resistance in bacteria [21]. Chitosan (CH) is an organic, GRAS-status, non-toxic polymer derived from the deacetylation of chitin; a natural polysaccharide present as the main component of exoskeletons of

crustaceans [22]. CH is biodegradable and has been widely used as an antimicrobial coating and film-forming polymer on food products [23,24,25]. Previously, CH adipate and CH have shown to have antibacterial effect against *Salmonella* Typhimurium [26] and *Salmonella* Gallinarum [27]. In addition, research conducted by our collaborators has identified the potential of CH in reducing *Campylobacter jejuni* in chicken wingettes [28] thereby highlighting the potential of the compound as an antimicrobial in poultry products. Another natural compound β-Resorcylic acid (BR; 2,4-dihydroxybenzoic acid) is a phytophenolic compound widely distributed among the angiosperms and is a secondary metabolite that plays a key role in the biochemistry and physiology of plants [29]. Moreover, we have reported in vitro antibacterial effect of BR on cattle hide against *E. coli* O157:H7 in a previous study [30]. Both CH (CFR 180.1072) and BR (CAS RN no. 89-86 [Everything Added to Food in the United States]) are approved for use in foods by the U.S. Food and Drug Administration. In this study, we investigated the prophylactic efficacy of feed supplemented with CH and BR (separate and combinatorial) in reducing SE populations in cecum, crop and liver in 21-day old broiler chickens.

Materials and Methods

Ethics Statement

All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut.

Experimental Birds and Management

Day-old commercial, non-vaccinated, straight run broiler chicks (Ross x Ross) were procured from Burr Farms, CT, and were distributed into floor pens in the University of Connecticut avian isolation facility. The facility is equipped with provisions for age-appropriate temperature, floor space, light, and bedding. The birds were given *Salmonella*-free feed (Blue Seal Feeds Inc., Londonderry, NH) and water ad libitum.

Bacterial Strains and Culture Conditions

A four-strain mixture of SE isolated from chickens (obtained from the Connecticut Veterinary Diagnostic Medical Laboratory, University of Connecticut) was used to inoculate the birds. The isolates were SE-12 (chicken liver, phage type 14b), SE-21 (chicken intestine, phage type 8), SE-28 (chicken ovary, phage type 13a), and SE-31 (chicken gut, phage type 13a). Each strain was pre-induced for resistance to 50μg/ml of nalidixic acid (NA; Sigma Aldrich, St. Louis, MO, USA) for selective enumeration [31,32]. One-hundred microliters of each NA-resistant strain was cultured separately in 10ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) overnight, transferred to flasks containing 100ml TSB supplemented with 50 μg/ml of NA, and incubated overnight at 37°C with shaking (100 rpm). Equal volume of each SE culture was combined and centrifuged at 3,600 x g for 15 min at 4°C. The pellet was washed and resuspended in 100ml of phosphate-buffered saline (PBS, pH 7.0), and used as the inoculum (10 log10 CFU/ML). The bacterial count in the individual cultures and the four-strain cocktail was confirmed by plating 0.1-ml portions of appropriate dilutions on xylose lysine desoxycholate agar (XLD; Difco) plates containing NA (XLD-NA) and incubating the plates at 37°C for 24h.

Experimental Design

Table 1: Experimental design.

Groups	Inoculated	Treatment
Positive control	SE	No BR/CH supplementation
0.5 or 1% CH treatment	SE	0.5 or 1% CH supplementation
0.5 or 1% BR treatment	SE	0.5 or 1% BR supplementation
CH and BR combination at 0.5 or 1%	SE	CH and BR combination supplemented at 0.5 or 1%
Negative control	No SE	No BR/CH supplementation
0.5 or 1% CH treatment	No SE	0.5 or 1% CH supplementation
0.5 or 1% BR treatment	No SE	0.5 or 1% BR supplementation
CH and BR combination at 0.5 or 1%	No SE	CH and BR combination supplemented at 0.5 or 1%

One hundred and sixty, straight run, day-old broiler chicks were weighed at the beginning of the trial, and randomly assigned to 2 replicates each for a control group (challenged with SE, no supplemental CH [Sigma-Aldrich, St. Louis, MO] or BR [Sigma-Aldrich]), low dose treatment (challenged with SE, 0.5% CH or BR), high dose treatment (challenged with SE, 1% CH or BR), combination of low doses of both compounds (challenged with SE, 0.5% CH and 0.5% BR) and a combination of high doses of both compounds (challenged with SE, 1% CH and 1% BR) (18 birds/group). The experimental design with appropriate negative controls is further explained in (table 1). On day 0, two birds from each experimental group were randomly selected and sacrificed to confirm that the birds were initially devoid of any *Salmonella*. In addition, cloacal swabs were collected from all birds prior to in-feed supplementation for *Salmonella* testing. CH and BR were supplemented in the feed for 20 days, starting on day 0. On day 8, birds in the positive control, CH and BR treatments were challenged with 1mL of the inoculum containing approximately

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8.0 log10 CFU of the 4-strain SE mixture by crop gavage. Cloacal swabs from all birds were analysed weekly until 20 days for the presence or absence of SE. After 13 days of challenge, 10 birds per treatment (n=10) were sacrificed by CO2 asphyxiation and cecum, liver and crop from each bird were collected for SE enumeration. Autopsy and tissue collection were performed at the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL), University of Connecticut. The samples were collected in 50 mL sterile tubes containing ice cold PBS, and were transported to the laboratory on ice for bacteriological analysis performed on the same day. This experiment was repeated three times in duplicates.

Determination of SE in internal organs and cloacal swabs

The presence of SE in the crop, liver and cecum were determined as described previously [31,32]. The organ samples and their contents from each bird were weighed and homogenized. Each homogenate was serially diluted (1:10) in PBS, and appropriate dilutions were plated on XLD-NA plates for bacterial enumeration. Representative colonies from XLD-NA plates were confirmed as *Salmonella* using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd. Camberley, UK). When colonies were not detected by direct plating, samples were tested for surviving *Salmonella* by enrichment in 100ml selenite cysteine broth (SCB; Oxoid) for 48h at 37°C, followed by streaking on XLD-NA plates. In addition, cecal pH was measured and cecal endogenous bacteria were enumerated by plating appropriate dilutions of the ceca samples on duplicate thioglycolate agar plates (TGA; Difco), followed by incubation at 39°C under 5% CO2 for 24h. The cloacal swabs were collected and mixed in 10ml of PBS followed by appropriate dilution and plating XLD-NA plates. As described above, representative colonies from XLD-NA plates were confirmed as *Salmonella* using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd. Camberley, UK). When colonies were not detected by direct plating, samples were tested for surviving *Salmonella* by enrichment in 100ml selenite cysteine broth (SCB; Oxoid) for 48h at 37°C, followed by streaking on XLD-NA plates.

Body Weight and Feed Consumption

The feed consumption and body weight of birds were determined for each experiment. Birds were weighed individually at the start and end of each experiment and averaged. The average feed consumption per bird was calculated by dividing the total amount of feed consumed per group by the number of birds in the respective group.

Statistical Analysis

A completely randomized design (CRD) was used to analyze the effect of CH and BR on SE in 21-day old broiler chicks and the experimental unit was pen. The experiment was repeated three times with duplicates for each treatment. The samples from which no bacteria were recovered after spread plating, but positive after enrichment were assumed a value of 0.91 for analysis (9cfu/mL) [33,34]. The data were analysed using the PROC-GLM procedure of the statistical analysis software (version 9.4, SAS Institute Inc. Cary, NC). Differences among the least square means were detected using Fisher's Least Significance Difference (LSD) test. A P-value <0.05 was considered statistically significant.

Results

Effect of CH and BR on SE colonization of internal organs

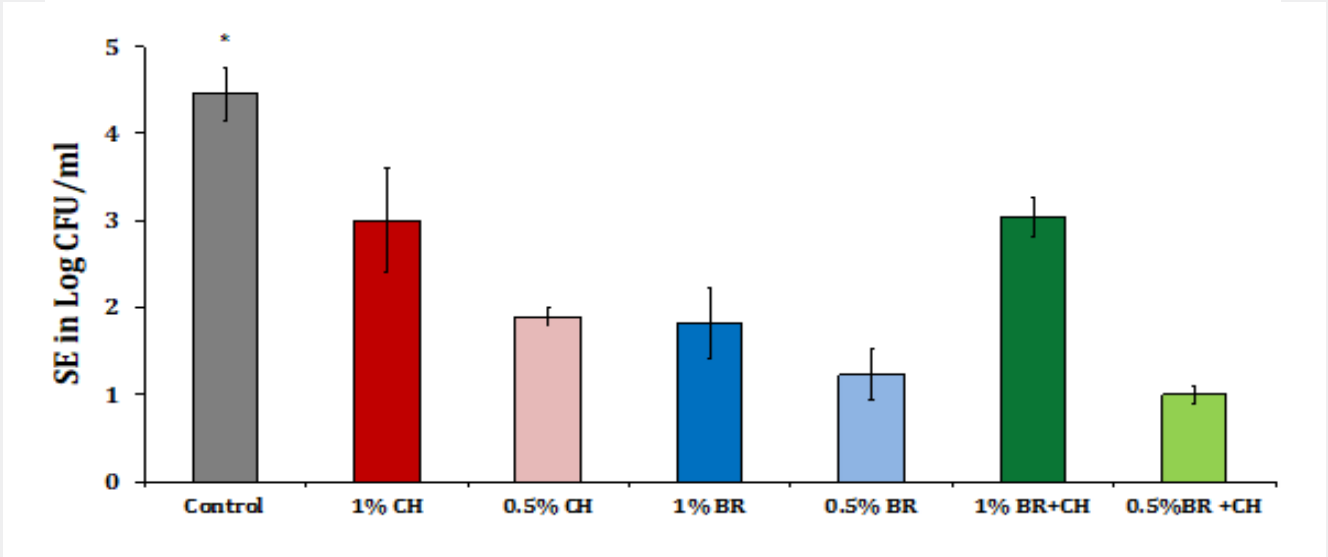

Figure 1a: Cecum

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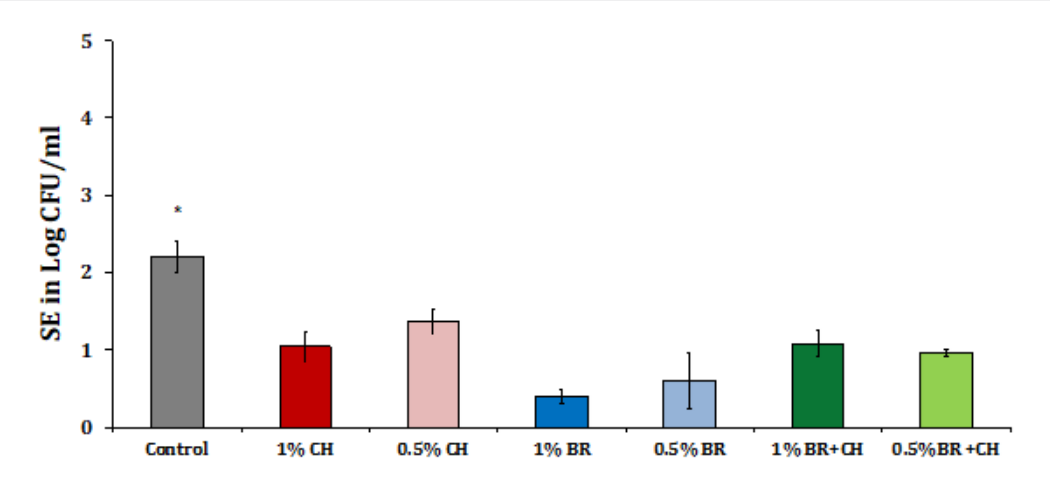

Figure 1b: Liver

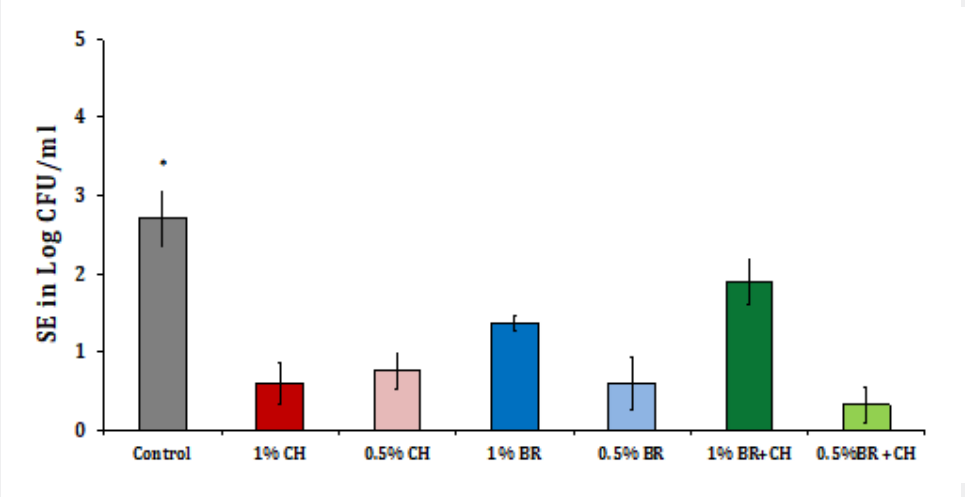

Figure 1c: Crop

Figure 1: Effect of CH or BR on SE in (a) cecum (b) liver (c) crop in 21-day old broilers. Error bars represent SEM. Grey column represents control treatment with *denoting significant difference between the treatments (P < 0.05) when compared with controls. Negative and compound controls were not included in the statistical analysis since SE was not recovered from those treatments.

Supplementation of CH and BR at 0.5% and 1% alone and in combination reduced (P < 0.05) SE counts in the cecum (Figure 1a), liver (Figure1b) and in crop (Figure 1c). The SE counts recovered from the cecal samples of the control birds ranged from 4 to 5 log10 CFU/g. CH at 1% reduced cecal SE by ~1.5 log10 CFU/g, whereas BR (1%) reduced SE by ~2.5 log10 CFU/g (P < 0.05). The combination treatment containing BR and CH at 1% was effective in reducing cecal SE by ~1.5 log10 CFU/g (P < 0.05). However, the combination with lower concentration of 0.5% CH and BR was more effective in reducing SE by ~ 3 log10 CFU/g (P < 0.05, Figure 1a). Similarly, BR (1%) and CH (1%) significantly decreased SE by ~ 2.0 and 1.5 log10 CFU/g (P < 0.05), respectively and their combination at 0.5 and 1% decreased SE by ~1.3 log10 CFU/g (P < 0.05) in liver, when compared to controls (Figure 1b). Consistent with the results from cecal and liver samples, CH (1%) and BR (1%) reduced SE in crop by ~2.3 log10 CFU/g and by ~1.3 log 10 CFU/g, respectively (Figure 1c). When compared to controls, combination of 1% BR and CH reduced SE by ~1 log 10 CFU/g (P < 0.05). Moreover, 0.5% combination reduced SE by ~2 log10 CFU/g (Figure 1c). Although all concentrations of CH and BR decreased SE colonization in the birds, there was significant difference (P < 0.05) in SE counts between 0.5 and 1% treatment groups.

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Body Weight and Feed Consumption

The average body weight of birds did not differ significantly between most of the groups (P > 0.05) for 21 days (Table 2). However, there was a significant difference (P < 0.05) between the average body weight of birds fed with 1% CH and 1% BR when compared to controls in the SE positive group and birds fed with 1% BR in comparison to the control in the SE negative group. The mean body weight (kgs) of positive controls were 0.60; whereas birds in the positive group fed with 1% CH and 1% BR had an average body weight of 0.54 and 0.53 kgs respectively (Table 2). The average feed intake was similar among the groups in all trials within the respective positive and negative group (P > 0.05, Table 2).

Table 2: Effect of Chitosan and β –resorcylic acid (wt/wt) on cecal pH, cecal endogenous bacteria, body weight and cumulative feed consumption of 21-day-old chickens (n = 18 per treatment, repeated thrice)

Treatments	Cecal pH	Cecal bacteria (log CFU/ml)	Average Body wt (Kg)	Cumulative feed intake (g)
Positive control	6.4±0.02	9.0±0.2	0.600**±0.052	705±6.2
0.5 % CH	6.4±0.02	8.8±0.2	0.629±0.02	724±3.2
1.0 % CH	6.45±0.02	8.7±0.2	0.543*±0.035	680±9.5
0.5 % BR	6.3±0.02	9.0±0.1	0.593±0.005	695±3.4
1.0 % BR	6.35±0.02	8.7±0.2	0.525*±0.04	685±9.8
0.5 % CH + 0.5% BR	6.3±0.02	8.6±0.2	0.595±0.01	700±3.2
1.0 % CH + 1.0% BR	6.35±0.02	8.8±0.2	0.586±0.01	693±6.2
Negative control	6.3±0.02	8.4±0.4	0.650##±0.025	752±2.2
0.5 % CH	6.4±0.02	8.8±0.2	0.645±0.03	750±2.2
1.0 % CH	6.3±0.02	8.5±0.3	0.600±0.015	728±4.0
0.5 % BR	6.2±0.02	8.5±0.2	0.655±0.02	750±5.7
1.0 % BR	6.15±0.02	8.5±0.2	0.571#±0.02	700±5.3
0.5 % CH + 0.5% BR	6.25±0.02	8.9±0.2	0.643±0.01	765±3.1
1.0 % CH + 1.0% BR	6.2±0.02	8.5±0.2	0.602±0.02	742±2.5

*- Treatments are significantly different from controls (**), P < 0.05,

- Treatments are significantly different from controls (##), P < 0.05.

Discussion

Cecum is the primary site of SE colonization with cecal carriage of pathogen leading to systemic contamination in chickens [35,36]. Besides the ceca, crop is another important site for pathogen survival and dissemination into external environment [36-38]. In addition, liver is also implicated during infection due to uptake of SE by macrophages and dissemination to the lymphatic system [35,36]. Therefore, in the current study, we investigated the efficacy of chitosan and β –resorcylic acid in reducing SE populations in the above-mentioned internal organs. Results revealed that all the concentrations of CH and BR either alone or in combination when supplemented through the feed were effective in reducing SE populations in cecum, crop and liver. Although both compounds reduced *Salmonella* counts in all the tested sites (P < 0.05), its effect was more pronounced in the cecum (Figure 1a) with a maximum reduction of ~3.5 log10 CFU/g in SE counts by 0.5% BR. Additionally, CH at 0.5% decreased the pathogen population by ~2 log10 CFU/g in the cloaca (Figure 1a). Also, BR was slightly more effective than CH in decreasing SE populations in liver with 1% BR reducing SE populations by ~2.5 log CFU/g (P < 0.05). These reductions in *Salmonella* counts in the cecum and liver are important for the microbiological safety of poultry products since high bacterial number in these two sites can cause both horizontal and vertical transmission of the pathogen leading to carcass and egg contamination [35,36,38]. Similarly, *Salmonella* recovery from the crop in controls was ~ 2.7 log CFU/g and in comparison, all treatments significantly lowered the SE count, with 0.5% BR and CH combination being the most effective combination reducing the pathogen to ~ 0.3 log CFU/g (P < 0.05) as depicted in (Figure 1c). The higher concentrations of BR and CH were slightly less effective alone and in combination in reducing the colonization in cecum (Figure 1a). This could be attributed to the decreased body weight (Table 2) in the groups due to supplementation of 1% BR and 1% CH. The mechanism of action of chitosan has been well studied due to its widespread use as food wraps in food safety [39-41]. One proposed mechanism of action is the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes which leads to altered cell permeability and the leakage of proteinaceous intracellular constituents [42-44]. In addition, chitosan also acts as a chelating agent, which can selectively bind trace elements thereby inhibiting toxins and bacterial growth [45]. Moreover, our collaborators have observed that CH, and its

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combination with phytochemicals can potentially modulate transcription of several genes essential for survival and virulence of *C. jejuni* [28]. In this regard, the use of CH as a pre-harvest feed supplement in chicken against food-borne pathogens such as SE has been minimally studied. Moreover, unlike CH, very little information is available on β-resorcylic acid as an antibacterial agent. BR lactones have been studied for their potential role in treatment for cancer and neurodegenerative diseases and can inhibit protein kinases and toxins [46]. However, the efficacy of BR in reducing food borne pathogens in animal system has been unexplored. Additionally, while recent studies have evaluated CH-based vaccines against SE [47,48], no research has investigated CH and BR as in-feed supplements for controlling SE in broilers. This manuscript presents the first study demonstrating the effectiveness of CH and BR as feed supplements in reducing SE colonization in broilers. The results from this study underscore the use of the two natural compounds as pre-harvest application to reduce the potential pathogen carriage in birds. This is critical as enteric contents containing the pathogen could contaminate the broiler carcasses during evisceration process and chilling [49] thereby compromising product safety.

Conclusion

The study demonstrates that BR and CH, used either individually or in combination, significantly reduced SE colonization in the cecum, liver, and crop of broiler chickens. Among the treatments, BR exhibited a slightly greater reduction in SE populations than CH, with the most pronounced effects observed at a 0.5% concentration. These findings underscore the potential of BR and CH as natural, safe, and pre-harvest feed additives to enhance food safety in poultry production. Further research is needed to elucidate their precise mechanisms of action against *Salmonella* and to optimize their application for commercial use.

Data Availability Statement

- The data presented in this study are available on request from the corresponding author. The data are not publicly available until publication of the manuscript.

Funding

This project was supported by the USDA Sustainable Agricultural Systems Grant Award Number: 2020-69012-31823.

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How to cite this article: Indu U, Abhinav U, Kumar V. B-Resorcylic Acid and Chitosan Reduce *Salmonella* in Broilers. Arch Anim Poult Sci. 2025; 3(1): 555604. DOI: 10.19080/AAPS.2025.03.555604

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