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Research Article
Effects of Organic Acid and Probiotics on Cecal Colonization and Immune Responses in Broiler Chickens Challenged with *Salmonella* Enteritidis

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Abstract

Objective: This study was designed to compare the effects of continuous supplementation of a pool of *Lactobacillus* and organic acid on the control of infection by *Salmonella* Enteritidis (SE) in broiler chickens. **Materials and Methods:** A total of 240 chickens were distributed in an entirely randomized experimental delineation into eight groups: G1: Basal diet, G2: Basal diet+challenge with SE, G3: basal diet+caprylic acid, G4: Basal diet+caprylic acid+SE, G5: Basal diet+5,7-dichloro-8-quinoline, G6: Basal diet+5,7-dichloro-8-quinoline+SE, G7: Basal diet+pool of *Lactobacillus*, G8: Basal diet+pool of *Lactobacillus*+SE. On the 4th, 14th, 24th and 36nd day post infection, blood was collected from the birds for immunological evaluation, ceca were collected for the microbiological evaluation of SE and quantification of interleukin 8 (IL-8) and cecal epithelial samples were collected for histopathological evaluation. **Results:** At slaughter, all the administered treatments demonstrated the capacity to reduce cecal colonization by SE, as evidenced by the microbiological and histopathological evaluations. The serum levels of IgM were not affected by the various treatments administered but rather only by SE challenge. IL-8 production was not affected by treatment or SE challenge. **Conclusion:** All the treatments evaluated here showed the capacity to control cecal colonization by SE in broiler chickens and suggest that these treatments may be employed as alternatives to the use of antimicrobials in the control of contamination by SE.

Key words: *Gallus gallus domesticus*, *Lactobacillus*, IgM, Interleukin, *Salmonella* Enteritidis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Worldwide, *Salmonella* constitutes one of the principal causes of diseases transmitted by food in humans and is responsible for substantial global causes of morbidity, mortality and economic losses[1]. Among the more than 2600 serovars of *Salmonella* described to date[2], *Salmonella* Enteritidis (SE) is recognized as one of the principal serovars responsible for cases of salmonellosis in human[3]. Poultry consumption has been identified as one of the principal causes of SE contamination among humans[4].

Another increasing public health concern is the development and transmission of antimicrobial resistance via the transfer of resistance genes between enterobacteria, including SE[5]. Although, currently not fully defined, this phenomenon has been partially attributed to the use of growth-promoting antimicrobials (GPA) in animal feed, which has led to restrictions against the use of certain antimicrobials in animal production[6]. The scientific community has been extensively researching alternatives to the use of GPAs, including organic acids, probiotics, essential oils and immunostimulants, that show a growth-promoting effect and an ability to reduce pathogen levels.

Among several antimicrobials used in poultry production, halquinol-a mixture of 5,7-dichloroquinoline-8-ol, 5-chloroquinolin-8-ol and 7-chloroquinolin-8-ol - is a potent non-antibiotic antimicrobial that possesses broad-spectrum antifungal, antibacterial (both gram-positive and gram-negative) and antiprotozoal activity[7]. Because halquinol is a non-antibiotic antimicrobial, it is generally believed that it does not cause bacterial resistance that may interfere with human therapeutics, thus making it an excellent alternative to GPAs.

In relation to organic acids and probiotics, several interventions have been investigated regarding the control of *Salmonella* Enteritidis at the field level, with varying degrees of success[8,9]. Medium-chain fatty acids (MCFA), including caprylic acid (CA), are reported to possess antibacterial activity against various microorganisms, including SE[9,10]. The present study sought to evaluate the effects of continuous supplementation of a pool of *Lactobacillus*, a non-antibiotic antimicrobial agent and a medium-chain organic acid on the control of SE infection in broiler chickens.

MATERIALS AND METHODS

The Ethics Committee on Animal Use of São Paulo State University (UNESP) approved all the procedures used in this experiment (Protocol Number 43/2013).

Strain of *Salmonella* Enteritidis and experimental challenge: The *Salmonella* Enteritidis (phage type 4) strain used in the experiments was isolated from Brazilian poultry farming. The strain was selected for nalidixic acid (Nal 100 μg mL^{-1}) and rifampicin (Rif 100 μg mL^{-1}) (Sigma-Aldrich, St. Louis, USA) resistance through successive passage on Brilliant Green Agar (BGA, Oxoid, Basingstoke, UK)

For the challenge, the strain was incubated in Brain Heart Infusion Broth (BHI; Oxoid, Basingstoke, UK) at 40°C for 18 h. The quantification of colony-forming units (CFU) was determined by serial dilutions in PBS at pH 7.2. At six days of life, the birds were experimentally challenged.

Birds, experimental treatments and additives: A total of 245 1-day-old male Coob® broiler chicks[11] were obtained from a private hatchery. The birds were distributed randomly in galvanized wire battery cages. The birds were vaccinated against Newcastle disease and Marek's disease. To ensure that the birds were free of *Salmonella* spp., meconium was collected prior to arrival and five birds were euthanized for subsequent *Salmonella* spp. research using the methodology of isolation recommended by Mallinson and Snoeyenbos[12].

Birds were initially maintained at 30°C and the temperature was gradually reduced by 3°C per week to 21°C by the end of week 3. This temperature was maintained for the duration of the experiment. Water and feed were supplied *ad libitum*. Birds were fed a corn and soybean meal-based diet free of antimicrobials and anticoccidial drugs. The nutritional levels followed the recommendations of the Cobb 500 Guides[11]. The raising period was 42 days and the feeding program was divided into three phases: starter (0-10 days), grower (11-22 days) and finisher (23-42 days).

During the experimental period, rigorous biosecurity procedures were maintained among the different groups to avoid cross-contamination between the different experimental groups.

Additives

Pool of *Lactobacillus* (Probiotic): The strains of *Lactobacillus* utilized in this study (*Lactobacillus plantarum*, *reuteri*, *acidophilus*, *brueckii* and *Lactobacillus* spp.) were isolated from broiler breeders and selected according to the adhesion capacity and immunomodulatory effects described by Rocha *et al.*[13] The strains were cultivated separately in 15 mL each of deMan, Rogosa and Sharpe broth (MRS, Acumedia, Lansing, USA) in anaerobic conditions at 37°C for 48 h and all strains were subsequently pooled (at 1:1:1:1:1 ratios). The inoculum containing 10^5 CFU mL^{-1} was administered orally via gavage daily to the birds in groups 7 and 8.

Organic acid: caprylic acid (≥99%): In liquid form (Sigma-Aldrich, St. Louis, USA) at 0.7% per kg of ration was added, throughout all life phases of the birds.

Antimicrobial growth promotor: A controlled mixture of 5,7-dichloro-8-quinolinol, 5-chloro-8-quinolinol and 7-chloro-8-quinolinol (60%)-Halquinol, a non-antibiotic antimicrobial agent- was added to 60 mg kg^{-1} of ration throughout all life phases of the birds.

For the antimicrobial substitution in the other diets, rice-husk meal (an inert supplement) was utilized.

Experimental design: The birds were distributed in a randomized experimental design into eight experimental groups of 30 birds each as follows: G1: Basal diet, G2: Basal diet + challenge with SE, G3: Diet supplemented with caprylic acid (0.7% per kg/ration), G4: Diet supplemented with caprylic acid (0.7% per kg/ration) +SE, G5: Diet supplemented with Halquinol (60 mg kg^{-1}), G6: Diet supplemented with halquinol (60 mg kg^{-1}) +SE, G7: Basal diet+pool of *Lactobacillus* (10^5 CFU mL^{-1} $bird^{-1}$) and G8: Basal diet+pool of *Lactobacillus* (10^5 CFU mL^{-1} $bird^{-1}$) +SE.

The birds in groups 2, 4, 6 and 8 were inoculated via oral gavage with 1 mL (10^7 CFU mL^{-1} of SE) per bird, respectively. The birds in groups 1, 3, 5 and 7 received 1 mL of PBS orally via gavage.

Parameters evaluated: On the 4th, 14th, 24th and 36nd day postinfection (dpi), six birds from each experimental group were randomly selected and euthanized. Collections were made of blood samples for immunological evaluation, the ceca for microbiological evaluation of SE and quantification of interleukin 8 (IL-8) and cecal epithelia for histopathological evaluation.

Quantification of immunoglobulin M levels: At 4, 14 and 24 dpi, 6 broilers per treatment were randomly selected for blood sample collection from the wing vein of the birds in heparinized tubes. Blood samples were conditioned in assay tubes for subsequent centrifugation (1.500×g, 5 min, 10°C) and isolation of serum, which was stored at -80°C. For antibody level determination, the technique of Enzyme Linked ImmunoSorbent Assay (ELISA) was utilized with a Chicken IgM ELISA quantification kit (Bethyl Laboratories, Montgomery, USA), following the manufacturer's instruction. The IgM levels were determined using a standard curve and values were expressed as nanograms per mL of serum.

Quantification of interleukin 8 (IL-8): To evaluate the concentrations of IL-8 in cecal fluid, one sample of the cecum was collected and, with the aid of a syringe, 2 mL of "washing solution" (PBS pH 7.0, 0.01% of thimerosal, 1% of BSA, 1 mM of phenylmethyl sulfonyl fluoride, 5 mM of EDTA) was injected into the proximal portion of the cecum so that the entire cecum would be washed. The fluid collected was centrifuged at 1.200x*g* for 7 minutes, permitting the separation of the supernatant, which was stored at -80°C.

The total IL-8 levels in the cecal wash were quantified via ELISA kit for IL-8 Chicken (*Gallus gallus*) (Uscn Life Science Inc., Wuhan, China), following the manufacturer's instruction. The levels of IL8 in the cecal fluid were determined using a standard curve and were expressed as nanograms per mL of cecal wash.

Quantification of *Salmonella* Enteritidis levels in the cecum: One of the cecum samples was removed, placed in an individual sterile plastic bag, weighed and macerated. According to the organ weight and contents, the quantity of PBS added was determined to reach a proportion of 1:10, obtaining the dilution 10^{-1}. After homogenizing the contents, 1 mL was removed to perform the remaining dilutions until reaching a concentration of 10^{-8} in test tubes containing 9 mL of PBS (pH 7.2). For plating, 0.1 mL of each dilution was plated in duplicate on BGA Nal/Rif plates and then incubated for 24 h at 40°C. The raw numbers for CFU g^{-1} of the contents and organs were converted to a $\log_{10}$ scale to interpret the results.

Histopathology: Samples of cecal tonsils and 2 cm segments from the medial part of the cecum were collected and immersed in 10% formalin. Dehydration of the tissues was followed by diaphanization via two xylene passages and soaking in plastic paraffin. The histological sections were stained with hematoxylin and eosin (HE), as described by Behmer *et al.*[14]. The samples were analyzed qualitatively regarding the evolution of the cecal tonsils and integrity of the cecal epithelium, with the aid of an image analyzer (Axio Vision) coupled to an optical microscope (Axio Imager A1, Zeiss).

Statistical analysis: In all the analyses, each bird was considered a biological repetition. The results of the SE count were subjected to logarithmic transformation to achieve normal distribution for subsequent analysis of variance complemented with Tukey's test analysis for multiple comparisons (5%), calculating the mean and standard error of

the mean (±SEM). The results of the IgM quantification in serum and the IL-8 determination in cecal fluid were compared via analysis of variance complemented with Tukey's test for multiple comparisons (5%)[15].

RESULTS

Quantification of *Salmonella* Enteritidis levels in the cecum: No birds in the non-challenged groups (G1, G3, G5 and G7) showed colonization by SE in the cecum. Clinical symptoms of SE infection were not observed. The colonization of the cecum by SE was affected by treatments at all timepoints evaluated (Table 1). Levels of cecal contamination in birds challenged but not treated (G2) were inversely proportional to the age of the birds.

Histopathology: The birds that were not treated or challenged (G1) did not show lesions in the cecal epithelium; neither did the birds that received caprylic acid (G3), Halquinol (G5) and *Lactobacillus* (G7). The birds in the groups challenged with SE showed different degrees of lesions in the cecal epithelium, with the birds in group G2 showing marked alterations, such as exposure of the villus apices and vascular congestion, which became increasingly sparse with advanced age.

In the cecal tonsils, there was greater intrinsic cellular proliferation of lymphoid follicles and increases in the number of follicles that compose the structure of lymphoid tissues of the tonsils in groups that were challenged with SE (G2, G4, G6 and G8) and in the *Lactobacillus*-treated group (G7) (Fig. 1).

Serum IgM Levels: The level of serum IgM was greater in SE-challenged birds, peaking on the 14th dpi (Fig. 2). The birds that did not receive SE challenge, independent of having received any type of treatment, showed IgM levels that were consistently lower.

Interleukin 8 levels: The levels of IL-8 in the cecal fluid did not vary between the challenged and non-challenged birds, independent of the administration of any of the treatments.

Table 1. Mean quantity of colony-forming units per gram (log_{10} CFU g^{-1} ± SEM) from the cecal contents of broiler chickens challenged orally with *Salmonella* Enteritidis

	*Cecum-CFU log_{10} g^{-1} ±SEM **Age (days postinfection)			
***Treatments	4 dpi	14 dpi	24 dpi	36 dpi
G2	6.2±0.3[a]	5.1±0.7[a]	3.0±0.3[a]	1.1±0.3[a]
G4	3.9±0.4[b]	3.0±0.2[b]	0.0[b]	0.0[b]
G6	4.1±0.2[b]	3.1±0.5[b]	0.2±0.3[b]	0.0[b]
G8	5.1±0.6[ab]	4.1±0.8[ab]	1.1±0.4[b]	0.0[b]
p-value	0.045	0.037	0.022	0.01

[a,b]Different letters in the column differ by the Tukey's test ($p<0.05$), *SEM: Standard error of the mean, **All birds were challenged with one dose of *Salmonella* Enteritidis (10^7 CFU mL^{-1}) at six days of life. ***Treatments: G2: Basal diet+challenge with SE, G4: Diet supplemented with caprylic acid +SE, G6: Diet supplemented with 5,7-dichloro-8-quinoline +SE, G8: Basal diet +pool of *Lactobacillus* (10^5 CFU mL^{-1}) +SE

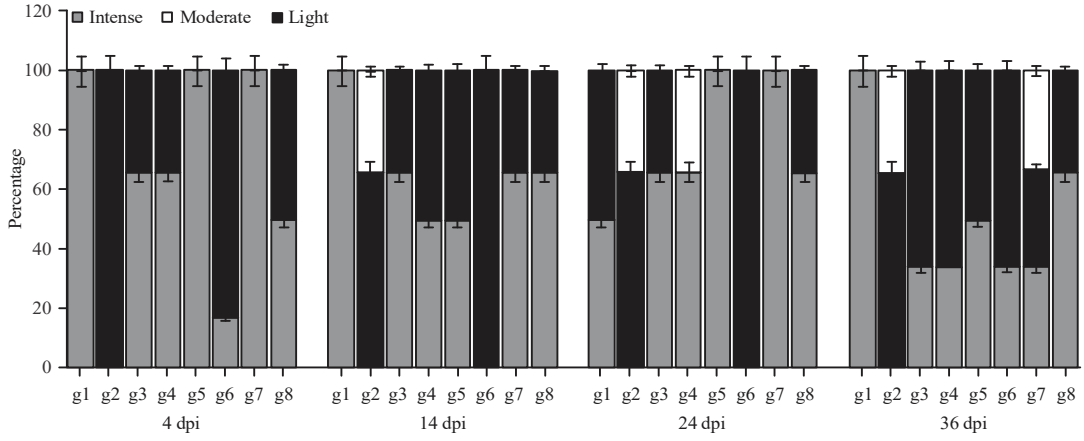

Fig. 1: Percentage of lymphoid proliferation in cecal tonsils of birds at 4, 14, 24 and 36 days after infection by *Salmonella* Enteritidis (SE), in the following groups: G1: Basal diet, G2: Basal diet+challenge with SE, G3: Basal diet+caprylic acid, G4: Basal diet+caprylic acid +SE, G5: Basal diet+5,7-dichloro-8-quinoline, G6: Basal diet+5,7-dichloro-8-quinoline +SE, G7: Basal diet+pool of *Lactobacillus*, G8: Basal diet+pool of *Lactobacillus* +SE

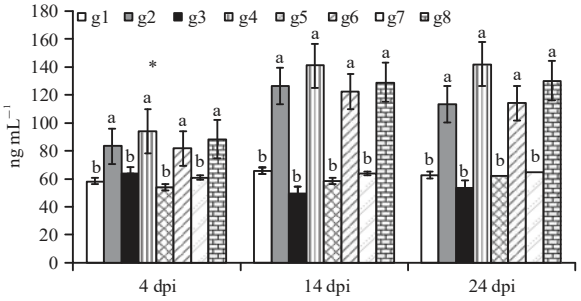

Fig. 2: Mean quantity of serum IgM levels at 6, 14 and 24 days after infection by *Salmonella* Enteritidis (SE), in the following groups: G1: Basal diet, G2: Basal diet+challenge with SE, G3: Basal diet+caprylic acid, G4: Basal diet+caprylic acid +SE, G5: Basal diet+5,7-dichloro-8-quinoline, G6: Basal diet+5,7-dichloro-8-quinoline +SE, G7: Basal diet+pool of *Lactobacillus*, G8: Basal diet+pool of *Lactobacillus* +SE

*a,bBars not sharing a letter are shown to be significantly different by the Tukey's test ($p<0.05$). Error bars represent the standard error of the mean. The value of n is 6

DISCUSSION

Salmonella spp. are pathogens that possess the capacity to colonize avian intestines and survive as transitory members of the intestinal microbial population and potentially causing serious disease. In most cases, the treating agent does not affect the carrier status of the bird, which is characterized by an asymptomatic infection, augmenting the probability of zoonotic transmission by means of the food chain[16]. In the present study, no clinical signs characteristic of infection were observed, despite the high cecal contamination found. Furthermore, it has been observed that, with increasing age in birds, there is a reduction of contamination independent of treatment, which is probably related to the development of the bird's immune system[17].

Medium-chain fatty acids (C6:0 to C12:0) have been utilized extensively for the control of *Salmonella* in broiler chickens. Currently, there are several combinations of organic acids available commercially, which can be coated or uncoated. Uncoated products are available in powder or liquid form and are administered in water or feed rations. According to Thompson and Hinton[18], the optimal action site after consumption of these acids is limited to the crop, due to the absorption that occurs subsequently throughout the intestinal tract. In the present study, even when employed in an uncoated form, caprylic acid demonstrated efficacious control of SE, reducing cecal contamination (at up to ~2.3 $\log_{10}$ CFU g^{-1}) and eliminating SE presence in the cecum after the 24th dpi. Furthermore, at a dose of 0.7% per kg of ration, the same treatment did not cause any type of pathological alteration in the cecal epithelium. In tests utilizing the same dose of caprylic acid and SE challenge, Kollanoor-Johny *et al.*[9] reported a quantitative reduction of SE not only in the cecum but also in the small intestine, cloaca, liver and spleen.

Several mechanisms of action have been attributed to the action of medium-chain organic acids, including caprylic acid. According to Bergsson *et al.*[19], caprylic acid can penetrate directly into cells and be incorporated into the bacterial plasmatic membrane, thereby altering their permeability. Furthermore, such acids can diffuse to the bacterial protoplasm and dissociate it, leading to intracellular acidification and, in this manner, adversely affecting bacterial enzymes and the transport of amino acids[20]. Several authors have reported that the action of organic acids is due to a reduction in pH or modulation of the intestinal microbiota[21]; however, it has already been demonstrated that caprylic acid, in its nonprotected form, does not alter the pH of cecal contents or the microbiota[10,22].

Previous studies have affirmed that several probiotic effects exerted by *Lactobacillus* spp are mediated by their interaction and adhesion to intestinal epithelium[23]. In this study, despite having utilized five previously selected strains of *Lactobacillus*, it was observed that they reduced the cecal contamination by SE only starting on the 24th dpi. It is known that the results of the action of probiotic products are variable and that comparisons between them are hampered due to the existence of many associated variables, such as the strains that compose the product, the route of administration, the viability of the product and the animal species in question[24].

According to Khan *et al.*[25], 8-hydroxyquinoline is a metal-chelating drug that can interfere with the metabolism of bacteria, fungi and protozoa. Furthermore, the halogenation of 8-hydroxyquinoline with chlorine derivatives accentuates the antimicrobial power of the compound. In the present study, we employed an antimicrobial derived from 8-hydroxyquinoline, composed predominantly of 5,7-dichloro-8-quinoline. At a dose of 60 mg kg^{-1} of feed, this product demonstrated the capacity to reduce cecal colonization by SE. Furthermore, the cecal epithelia of the birds that received it were found to be intact demonstrating that the product diminished the bacterial penetration into intestinal cells.

IgM is the first immunoglobulin synthetized in any specific immune response, with its levels being elevated between the fourth and fifth day after the initiation of the infection[26]. The birds that were not challenged with SE (G1, G3,

G5 and G7) showed lower serum levels of IgM when compared to the levels found in birds challenged with the bacteria. The infection caused by *Salmonella* can elevate the serum levels of immunoglobulins like IgM, IgG and IgA[27], as identified in the present study. Furthermore, it was verified that the IgM levels increased on the 14th dpi, remaining elevated until the 24th dpi. Similar results were also reported by Withanage *et al.*[27] while evaluating birds subjected to primary infection by *Salmonella* Typhimurium. According to Chart *et al.*[28], the serum levels of IgM are correlated with the route of inoculation and the challenge magnitude of SE infections, since elevated doses of bacteria administered orally induce higher serum titers of this immunoglobulin.

Probiotic bacteria, such as *Lactobacillus* spp., can modulate the immune response, stimulating the production of immunoglobulins[29]. Although, the strains utilized here have demonstrated prior immunomodulatory capacity[13], no positive effect on the production of serum IgM was found. It is important to emphasize that the immunomodulatory effects demonstrated previously were restricted to secretory IgA and IgG, with no positive effect on the production of these immunoglobulins in the serum being demonstrated[13]. These results contrast with those of other studies, in which the administration of probiotics provoked augmentation of the immune response of chickens against specific antigens[30,31] but agree with results reported by Mountzouris *et al.*[8], which did not show a positive effect of the use of probiotic product on the humoral immune response in broiler chickens. One question that must be considered in evaluating the results of the actions of probiotic strains is whether they were administered orally via gavage with a daily frequency. This activity may have induced stress in the birds and subsequent production and release of corticosterone, a glucocorticoid hormone released in situations of stress with the capacity to dysregulate the responses of the avian immune system[32].

IL-8 is a cytokine produced by diverse cell types, including epithelial and endothelial cells, with the capacity to attract neutrophils and T lymphocytes to an infection site[33]. Intestinal colonization by commensal microbiota, after avian hatching, may cause an inflammatory reaction due to a response of the local immune system that is characterized by an increase in IL-8 expression[34]. Furthermore, it is believed that two peaks of IL-8 expression occur in the cecum of healthy birds, one at four days of life and the other before the tenth day, after which there is a decline in the release of this interleukin. A study performed by Crhanova *et al.*[35] demonstrated that SE infection stimulates the expression of IL-8 at higher levels when compared to the stimulus from normal intestinal microbiota. In the current study, we did not observe any difference in the IL-8 levels in cecal fluid, independent of the presence of SE challenge or treatment. This result may be related to the high coefficient of variation obtained among birds of the same experimental group, evaluation timepoint (e.g., 4, 14, 24 and 36 dpi) or technique utilized.

CONCLUSION

All the treatments evaluated herein showed the capacity to control cecal colonization by SE in the final life phase of the birds, which signifies a crucial moment in the epidemiological transmission of the agent, as SE can contaminate the carcasses and reach the final consumer, thus causing serious public health challenges.

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