Inclusion of Organic Acids in the Drinking Water and Feed for the Control of Salmonella Heidelberg in Broilers

ABSTRACT

Poultry products may be a source of foodborne human salmonellosis. The use of alternatives to antimicrobials that are not harmful to humans may reduce the presence of Salmonella spp. in poultry production. Among the products used, organic acids stand out. In the present study, three different organic acid (OA) blends were evaluated for the control of Salmonella Heidelberg (SH) in commercial broilers. Day-old chicks (n = 114) were randomly assigned to four treatments, with three replicates of 12 birds each. Birds in treatments A and B received SCFA (0.2mL/L) and SCFA + MCFA (0.2mL/L), respectively, in the drinking water, while birds in treatment C received SCFA + MCFA in the feed (2g/Kg of feed). Birds from treatment D did not receive OAs (control group). At 8 days of age, each bird was orally inoculated with SH at 10^8 CFU/mL, and cloacal swabs and SH enumeration of the cecal content were performed 24-, 48-, and 72-hours post-inoculation (hpi). The results show a reduction of both SH shedding and counts in the birds fed OAs at all pi times relative to the control birds. Fecal shedding was significantly lower in the OA-treated groups compared with the control group. As for SH presence in the cecum, significant differences were detected between groups C and D at 24 and 72 hpi, and between groups B and D at 72 hpi. The results of this study indicate that the use of feeding OAs to broilers may contribute to reduce the incidence of SH in the poultry production chain, allowing better flock health management, provided an efficient biosecurity program is employed.

INTRODUCTION

Poultry products are relevant sources of human foodborne salmonellosis. Among Salmonella spp. serovars associated with human foodborne infections carried by poultry products, Salmonella Heidelberg (SH) has been one of the most frequently isolated in the last decade (CDC, 2013; Gieraltowski et al., 2016; Green et al., 2018; IFSAC, 2019).

Bacteria of the genus Salmonella can be introduced in commercial poultry farms by infected day-old chicks or by the consumption of contaminated feed (Berchieri Junior et al., 1989; Zancan et al., 2000). When broilers are contaminated with Salmonella spp. at hatch or at an early age, their immune system is still immature, allowing their gastrointestinal tract (GIT) to be readily colonized, resulting in long periods of microorganisms fecal shedding, and may cause systemic infection (Freitas Neto et al., 2020). In addition, Salmonella spp. may persist and spread in poultry farms carried by rodents, wild birds, and insects, such as the lesser mealworm (Alphitobius diaperinus) and the house fly (Musca domestica) (Crippen et al., 2009). These factors may explain why it is difficult to eliminate them from poultry production (Andino & Hanning, 2015).
Despite the many measures taken to try to prevent *Salmonella* gut infection of commercial poultry, its occurrence is still frequent. The use of feed additives that are not harmful to humans can help minimize its presence in farms. In particular, probiotics, prebiotics, phytogenics, and organic acids stand out (El Baaboua et al., 2018; Khan & Chousalka, 2020). When associated with good management practices and biosecurity measures, feed additives can be very efficient, especially organic acids (OAs) (Oliveira et al., 2000; Borsoi et al., 2011a; Pickler et al., 2012; Zabot et al., 2018; Calaça et al., 2019).

Organic acids are described as performance and intestinal health enhancers in broilers (Viola & Vieira, 2007; Calaça et al., 2019). Their antimicrobial action is attributed to their capacity to reduce the pH of GIT and directly act on the cell wall of Gram-negative bacteria, resulting in bacteriostatic or bactericidal effect (Dittoe et al., 2018). Although the main benefits of OAs are related to GIT pH reduction, these compounds can also prevent the spread of pathogens by penetrating their cell wall, which is sensitive to external pH variations, and changing their physiology, such as the case of enteric serovars of *Salmonella* spp. (Van Immerseel et al., 2003).

Organic acids used as feed additives are classified as short-chain fatty acids (SCFA), represented by formic, acetic, propionic and butyric acids, and medium-chain fatty acids (MCFA), such as capoic, caprylic, and capric acids. In their non-dissociated form, OAs of both classes change bacterial physiology, whereas MCFAs also reduce the expression of virulence genes in bacteria, impairing their capacity to invade intestinal epithelial cells (Van Immerseel et al., 2004; Rubio et al., 2009). Here, we presented an *in-vivo* research using three commercial organic acid blends added to the drinking water and feed of commercial broilers, aiming to evaluate the efficacy of these compounds to control *Salmonella* Heidelberg in broilers GIT.

**MATERIAL AND METHODS**

The study was carried out in the Avian Pathology sector of FCAV/Unesp, Jaboticabal campus, in accordance with the Ethical Principles on Animal Experimentation developed by the Brazilian College of Animal Experimentation and approved by the internal Ethics Committee on the Use of Animals (CEUA Process 012807/19; approved on 10 October 2019).

**Inoculum preparation**

The inoculum was prepared using a field isolate of *Salmonella* Heidelberg resistant to nalidixic acid and spectinomycin (SH-NalSpc), belonging to the bacterial library of the Avian Pathology sector, FCAV/Unesp. The strain is stored at -80 °C in lysogeny broth (LB; Sparks, Maryland, USA) supplemented with 30% glycerol and was seeded in 10 mL of LB broth and incubated at 37°C for 18 h at 150 revolutions per minute (rpm).

**In-vivo assay**

Day-old broiler chicks were obtained from a commercial hatchery and housed, immediately after arrival, in experimental cages (36 birds per cage), equipped with trough feeders and pressure drinkers, located in an air-conditioned room. The birds were offered water and feed *ad libitum*. The feed was based on corn and soybean meal and formulated to supply the birds’ nutritional requirements according to the genetic company manual with the following levels: 9,500 kcal of metabolizable energy/g of diet, 22.2% of crude protein, 1.31% of digestible lysine, 0.852% of digestible threonine and 0.94% of digestible methionine + cystine. The feed did not contain any antimicrobials, anticoccidials, or animal meals.

In order to confirm that day-old chicks were free from *Salmonella* spp. at housing, drag swabs of the chick transport crates were collected as described by Zancan et al. (2000). In brief, a sterile cotton gauze soaked in Buffered Peptone Water (BPW) (Oxoid®, Basingstoke, Hampshire, UK - CM0509) was dragged on the meconium present in the chick transport crate, placed in a flask containing 50 mL Selenite broth (SN) (Oxoid®, Basingstoke, Hampshire, UK - CM0395) supplemented with novobiocin (4mg/mL), and incubated at 37 °C for 24 hours. Using a bacteriological loop, the SN broth then was plated on Brilliant Green agar (BG) (Oxoid®, Basingstoke, Hampshire, UK - CM0263) and MacConkey agar (MC) (Oxoid®, Basingstoke, Hampshire, UK - CM0115). The plates were incubated at 37 °C for 24 h, and the presence of suggestive colonies of *Salmonella* spp. was evaluated.

**Experimental SH challenge and design**

At 8 days of age, each bird was challenged once with 0.5 mL of the previously prepared SH-NalSpc inoculum containing 10⁸ CFU/mL, which was administered directly into the crop with an intra-esophageal cannula. The treatments were applied between 6 hours post-inoculation (hpi) and 72 hpi. The evaluated products¹ included a blend of formic acid and propionic acid.
(SCFA) in the liquid form and a blend of formic acid and propionic acid combined with caprylic acid and capric acid (SCFA + MCFA) in liquid and powder form.

A total number of 144 broilers were randomly assigned to four treatment groups (A, B, C, or D), with three replicates of 12 birds each: in treatment A, the birds received SCFA in drinking water (0.2mL/L); in treatment B, SCFA + MCFA in the drinking water (0.2mL/L); in treatment C, SCFA + MCFA in the feed (2g/kg of feed), and birds in treatment D did not receive OAs (control treatment). The description of the treatments is shown in Table 1. Drinking water pH was measured before the beginning of the experiment and after the products were added.

**Bacteriological assays**

At 24, 48, and 72 hpi, four birds per replicate (12 birds per treatment) were sacrificed by neck dislocation for the collection of cecal contents. In order to determine possible SHNalSpc shedding, cloacal swabs were collected immediately before euthanizing and placed in tubes containing 3 mL SN broth. After homogenization, swabs were streaked on Bright Green agar with 100µg/mL of nalidixic acid and spectinomycin (BG-Nal/Spc) and incubated at 37 °C for 24 h. In the absence of colonies after this period, the broth was again incubated at 37 °C for 24 h and plated under the same conditions.

SHNalSpc in the cecal content was enumerated according to the method proposed by Barrow et al. (1987). In brief, the cecal content was diluted in Buffered Saline solution (PBS) at pH 7.4 at a ratio of 1:10, followed by serial decimal dilutions in tubes containing PBS at pH 7.4, which were plated on BG-Nal/Spc agar. Plates were read after incubation at 37 °C for 24 h. When the presence of SHNalSpc was not detected after this period, an equal volume of SN broth at double concentration was added to the broth tubes. The tubes were incubated at 37 °C for 24 h, and the broth was then plated on BG-Nal/Spc agar. The number of CFU/g was transformed into log_{10} for statistical analysis and interpretation of the results.

**Statistical analysis**

Data on fecal shedding of SHNalSpc, obtained from cloacal swabs of A, B, C, and D groups, were analyzed by the non-parametric Chi-Square Test at 5% of significance level (Zar, 2010). SHNalSpc enumeration (CFU/g) in the cecal content were logarithmically transformed and subjected to analysis of variance (ANOVA) followed by the Tukey Test at 5% of probability level (p<0.05). All statistical analysis were performed using the GraphPad Prism software for Windows, version 8.00 (GraphPad Software, La Jolla, California, USA).

**RESULTS**

The analysis of drag swabs of the chick transport crates did not demonstrate the presence of Salmonella spp. Moreover, the drinking water pH immediately before OA addition was 8.31 and after the addition of treatments A and B the pH levels decreased to 3.96 and 4.2, respectively.
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Figure 1 – Salmonella Heidelberg (SH) counts in the cecal of broilers challenged with SH and fed with organic acids (Group A: received SCFA in drinking water (0.2mL/L); Group B: SCFA + MCFA added to the drinking water (0.2mL/L); Group C: SCFA + MCFA in the feed (2g/kg of feed); and Group D did not receive OAs (control treatment). Different letters indicate statistical difference by the Tukey’s test (p<0.05).

Likewise, the control birds (Group D) showed higher SH counts in the cecal content relative to those fed the evaluated AO blends (Figure 1). Compared with the control treatment (Group D), a significant reduction of the bacterial load was determined in Group C (p<0.05) at 24 hpi, whereas no statistical differences among treatments were detected at 48 hpi. At 72 hpi, lower SH counts were detected in Group B (p<0.01) and Group C (p<0.05) compared with Group D.

DISCUSSION

Avian salmonellosis is an important cause of concern for poultry farmers, both because the disease affects bird performance and health, and because poultry products are often associated with foodborne human salmonellosis (Chittick et al., 2006; IFSAC, 2019). Controlling or minimizing the presence of paratypical Salmonella species is not an easy task, as its shedding in the feces contaminates the environment and allows its spread on poultry farms. According to Freitas Neto et al. (2020), measures to prevent Salmonella infection in young birds reduce its fecal shedding, contributing to the presence of these pathogens in the poultry production environment.

The dietary inclusion of performance-enhancing antimicrobials or antibiotic growth promoters (AGP) was of the main measures applied to mitigate the negative impacts of salmonellosis on the gastrointestinal tract of poultry for many years (Calaça et al., 2019). However, the in-feed inclusion of AGPs has been banned in European countries because they may promote the emergence and selection of resistant microorganisms of public health importance, leading to consumer demands for animal products free from antimicrobial residues (COUNCIL, 2003; Seleha et al., 2009). An alternative is the inclusion of organic acids (OAs) due to their antimicrobial potential (Van Immerseel et al., 2007; Pickler et al., 2012). The inclusion of alternative feed additives, such as probiotics and organic acids, in the drinking water or in the feed, may aid reducing Salmonella contamination of poultry farms as part of a comprehensive biosecurity program (Borsoi et al., 2011a).

The results of the present study support literature findings indicating that the acidification of drinking water and of feeds using OAs contributes to the control of the spread of agents of paratypical infections, reduces fecal bacterial shedding, and improve the performance of broilers (Byrd et al., 2001; Jarquin et al., 2007; Pickler et al., 2012; Machado Junior et al., 2014). Other studies also report the capability of OAs to decrease Salmonella spp. fecal shedding and colonization levels of the internal organs of infected poultry (Thompson et al., 1997; Menconi et al., 2013), and suggest that investments in OAs are cost-effective in broiler production. According to Al-tarazi & Alshwabkeh (2003), the combination of formic acid with propionic acid (SCFA) can also effectively control systemic infection, as demonstrated by the reduced mortality and colonization of the crop and cecum of laying chicks challenged with S. Pullorum.

The most accepted antimicrobial modes of action of OAs are related to the diffusion of its undissociated form through the membrane of microorganisms and reduction of intestinal pH (Cherrington et al., 1991). Upon entering the microbial cell, acids dissociate, suppressing cell enzymes and nutrient transport systems. These actions are dependent on OAs chemical formula and form, molecular weight, minimum inhibitory concentration against specific bacteria, as well as on microbial pH range and their nature (Huyghebaert et al., 2011). Therefore, combinations of different organic acids may have a broader spectrum of activity, as observed in the present study.

Although treatments A (SCFA) and B (SCFA + MCFA) reduced drinking water pH from 8.31 to 3.96 and 4.2, respectively, resulting in lower cecal colonization by SH compared with the control treatment, the in-feed inclusion of SCFA + MCFA (Group C) proved to be more effective (p<0.05) in controlling SH spread in broilers. This result may be attributed to the antimicrobial activity of OAs against Salmonella spp. Organic acids, even at low concentrations, enhance gut acidification and remain longer in the intestinal tract of broilers when included in the feed than in the drinking water (Nakai et al., 2003; Van Immerseel et al., 2011a).
al., 2004). It should be noted the observed differences in cecal SH counts between the treatments including MCFA+SCFA compared with the treatment with only SCFA suggest a synergistic effect between these acids, hindering the colonization and invasion of intestinal epithelial cells by suppressing the hilA gene, which regulates the pathogenicity island I of bacteria of the genus Salmonella (Baxter & Jones, 2015).

The results of this study suggest that the in-feed inclusion of organic acids aids in the control of SH in broiler farms, particularly when applied early in the grow-out cycle, as it was shown that day-old chicks are already frequently infected at arrival on the farm (Zancan et al., 2000; Freitas Neto et al., 2020) and that SH is detected in the cecum as soon as 6 hours after infection (Bossoi et al., 2011b). Therefore, in-feed organic acid blends may contribute to enhance the control of SH spread in broiler farms as part of an adequate biosecurity program.

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