Use of blends of organic acids and oregano extracts in feed and water of broiler chickens to control *Salmonella* Enteritidis persistence in the crop and ceca of experimentally infected birds[1]

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Primary Audience: Veterinarians, Nutritionists, Flock Supervisors, Quality Assurance Personnel, Researchers

SUMMARY

The aim of the present evaluation was to assess the efficacy of blends of organic acids and oregano extracts supplied from d 1 to 21 and from d 35 to 42 in feed, drinking water, or both, in controlling *Salmonella* Enteritidis persistence in the crop and ceca of broiler chicken. A total of 105 one-day-old male broiler breeder chicks were randomly distributed into 5 different treatments according to the supply of referred blends and *Salmonella* Enteritidis challenge. *Salmonella* Enteritidis was inoculated directly in the crop of birds at the 15 d of age. Treatments were unsupplemented unchallenged, unsupplemented challenged, supplemented through water and challenged, supplemented through feed and challenged, and supplemented through feed and water and challenged. Use of the additives in feed and water and in water alone efficiently controlled *Salmonella* shedding and reduced cecal persistence. Immune mechanisms involved are proposed.

Key words: organic acid, *Salmonella*, natural extract, broiler chicken, flow cytometry

2014 J. Appl. Poult. Res. 23:671–682
http://dx.doi.org/10.3382/japr.2014-00979

DESCRIPTION OF PROBLEM

Salmonella are gram-negative bacteria commonly inhabiting the gastrointestinal tract of various warm-blooded animals. Among the different subspecies, *Salmonella enterica* is the most common cause of domestically acquired food-borne bacterial illness in the United States

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and the serotype Enteritidis is the most common cause of salmonellosis [1]. In the southern region of Brazil, *Salmonella* Enteritidis was also the most commonly isolated pathogen from cases of human food-borne diseases [2–4]. Many authors link most human cases of *Salmonella* Enteritidis infection to the consumption of chicken meat or undercooked eggs [1, 5, 6]. *Salmonella* Enteritidis, though, is not commonly related to clinical disease in adult chickens, causing asymptomatic intestinal infections in birds, especially when animals are infected after the second week of life [7, 8], which may facilitate the spread of the pathogen among flock. Due to these factors, controlling *Salmonella* Enteritidis within the chicken-production system is of major concern. Such control can be implemented through multiple approaches, such as preventive hygienic measures and vaccination of poultry breeder flocks, use of genetically resistant chicken lines, acidification of feed and drinking water with short- and medium-chain fatty acids, as well as modification of the nutrient composition of the diet to discourage *Salmonella* infection and persistence in chicken organisms, and also adoption of optimal slaughtering and processing conditions [9].

Among the farm-level strategies to avoid *Salmonella* infection and persistence in broilers, the use of short-chain fatty acids through the feed or drinking water has been described as an effective alternative [10–14]. However, some factors, such as the mode of administration and the type and concentration of acids, are of major concern to effectively control *Salmonella* colonization in birds [15]. In addition, different serovars of *Salmonella* may respond differently to the action of organic acids in vivo [16].

Other compounds gaining importance regarding improvement of broiler gut health parameters are the essential oils (**EO**), mainly derived from plants, and therefore also called natural extracts. Beneficial effects have been reported by several authors evaluating blends of different compounds such as thymol and carvacrol [17]; thymol, carvacrol, eugenol, curcumin, and piperin [18]; carvacrol, cinnamaldehyde, and capsaicin [19]; eugenol [20]; EO from citrus, oregano, and annase [21]; and carvacrol, anethol, and limonen [22]. Supplying eugenol [20] through the feed of layers leads to a preventive effect regarding *Salmonella* cross-contamination in eggs. Therefore, the combination of organic acids (**OA**) and EO may offer beneficial effects in the control of *Salmonella* within the broiler chicken production cycle, as demonstrated in a previous study [23], where a significant reduction in *Salmonella* Enteritidis shedding from birds infected with the bacterium and treated with a blend of OA and EO was observed. Those authors also reported the need for a constant feeding of such blend to prevent shedding. As *Salmonella* Enteritidis may persist in the broiler flock when infection occurs after the second week of life, the present work was carried out to evaluate the effect of 2 blends of OA and oregano extract (**OE**) supplied in the feed, drinking water, or both, on *Salmonella* Enteritidis recovery and persistence in the crop and ceca during the whole rearing cycle of broiler chickens experimentally infected with the pathogen on d 15 posthatch.

MATERIALS AND METHODS

Birds, Diets, and Handling Procedures

A total of 105 one-day-old male broiler breeder chickens, Cobb 500, were randomly distributed into 5 different treatments of 21 birds each, in 5 identical rooms (2 m^2), located side by side but physically isolated. All rooms were covered with ceramic tiles (walls and floor, the latter covered with a wood shavings bed) and equipped with negative pressure ventilation system with high-efficiency particulate air (HEPA) filters to prevent any cross contamination. A mash corn and soybean-based feed was provided to all treatments. Three different diets were adopted according to the rearing phase, to meet or exceed the requirements for the species [24]. During the entire experimental period, birds were kept under optimal housing conditions and feed and water were provided ad libitum.

Each room with 21 birds corresponded to a different treatment, characterized by *Salmonella* Enteritidis infection and the supply of a blend of OA and OE via drinking water and or feed (Table 1). Treatments were negative control (**NC**), receiving feed and water without additives (acids or antibiotics), not challenged with *Salmonella* Enteritidis; positive control (**PC**), receiving feed and water without additives, challenged with *Salmonella* Enteritidis; organic acids in

feed (**OAF**), receiving feed supplemented with 0.2% of a blend of powder OA and OE [25] during the starter (1–21 d) and finisher (35–42 d) phases, challenged with *Salmonella* Enteritidis; organic acids in water (**OAW**), receiving feed without additives and water supplemented with 0.08% of a blend of liquid OA and OE [26] during the starter (1–21 d) and finisher (35–42 d) phases, challenged with *Salmonella* Enteritidis; and organic acids in feed and water (**OAFW**), receiving water and feed supplemented with 0.08 and 0.2% of liquid and powder blends of OA and OE, respectively, during the starter (1–21 d) and finisher phases (35–42 d), challenged with *Salmonella* Enteritidis.

Both blends of OA and OE had as active ingredients mainly formic, lactic, and acetic acids, as well as carvacrol. The solution of water and acids offered to treatments OAW and OAFW was prepared 1.5 h before being administered to birds, as per the recommendations of the supplier. At housing and once a week, all birds and feed were weighted and data were recorded to control BW gain and feed consumption. Birds were watched daily for the occurrence of any clinical signs related to *Salmonella* Enteritidis infection.

Salmonella Inoculation

Salmonella Enteritidis inoculum was prepared with isolates previously cultured from broilers. Isolates were streaked in seed stock agar and incubated in brain heart infusion solution for 24 h at 37°C. The solution was diluted to a 0.5 MacFarland concentration, corresponding to a concentration of 10^8 cfu/mL of *Salmonella* Enteritidis. Serial dilutions were performed in tubes containing 9 mL of sterile saline solution to a concentration of 10^5 cfu/mL. On d 15 posthatch, all birds (except those from NC) were inoculated with 1 mL of the *Salmonella* Enteritidis solution at a concentration of 10^5 cfu/mL via gavage.

Sampling

On d 1 after hatch, 5 birds were euthanized through cervical dislocation and the liver and cecum were collected and evaluated for the presence or absence of *Salmonella*. Prior to the beginning of the experiment, rooms were cleaned and disinfected and *Salmonella* analysis was conducted to certify the environment was not contaminated. On d 7 posthatch, cloacal swabs were taken from all birds for *Salmonella* analysis.

On d 14, 22, and 42 posthatch, 3 mL of blood was collected from the brachial wing vein of 8 birds from each treatment to quantify blood leucocytes through flow cytometry evaluations. All blood collections were made using sterile syringes and needles. After collection, blood was transferred to 10-cm glass tubes containing EDTA [27] as an anticlotting agent and stored under refrigeration until preparation for analysis.

At 48 h after challenge (d 17 posthatch) and on d 28 posthatch, cloacal swabs were taken from all birds (5 samples/treatment, each sample

Table 1. Treatment identification according to *Salmonella* Enteritidis challenge and the supply of organic acids and oregano extract in feed, water, or both

Treatment[1]	*Salmonella* Enteritidis infection (1×10^5 cfu/bird)	Organic acids and oregano extract
NC	None	None
PC	On d 15 posthatch	None
OAF	On d 15 posthatch	0.2% supplied in feed[2]
OAW	On d 15 posthatch	0.08% supplied in drinking water[3]
OAFW	On d 15 posthatch	0.2% supplied in feed + 0.08% supplied in drinking water

[1]NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

[2]Organic acids and oregano extract in feed (powder product): Acidal NC [25].

[3]Organic acids and oregano extract in water (liquid product): Acidal ML [26].

composed by a pool of 4 birds) and *Salmonella* Enteritidis analysis was performed. On d 22 and 42 posthatch, euthanasia and necropsy were performed in 8 birds per treatment. The entire crop and 1 cecum were collected for *Salmonella* Enteritidis analysis. To verify the presence of *Salmonella* in the environment, drag swabs were collected (1 sample per treatment) in each treatment on d 22, 28, and 42 posthatch.

Bacteriological Analysis and Flow Cytometry Evaluations

Salmonella analysis was performed on cloacal swabs, drag swabs, crop, and cecum samples as previously described [16]. Briefly, samples were homogenized in 1:9 of 2% buffered peptone water [28]. Further dilution was conducted by successively placing 1 mL of the solution in a test tube with 9 mL of 0.1% peptone water until a 10:3 dilution was achieved. Then, 100 μL of each dilution were transferred to duplicate plates in brilliant green agar base [29] and uniformly spread with a sterile Drigalsky loop. Plates were incubated at 35°C for 24 h after which the typical colonies were counted.

The initial 2% peptone water solution was also incubated for 24 h. If no typical *Salmonella* colonies had developed after the 24-h incubation, 100 μL of the initial 2% peptone solution were placed in a tube with 10 mL of Rappaport-Vassiliadis broth [30] and incubated at 42°C for 24 h to confirm the negative or positive results of samples [16].

The percentage of circulating macrophages, cluster of differentiation (**CD**) 8 and CD28, CD4 and T cell receptor (**TCR**) Vβ1, and B lymphocytes, as well as the CD4-to-CD8 ratio were quantified using the flow cytometry technique at Imunova Análises Biológicas (Curitiba, Brazil). Preceding flow cytometric analysis, mononuclear cells were separated from other blood compounds and then marked with specific antibodies according to a previously described technique [31]. Briefly, blood was diluted 1:1 with sterile PBS to a total volume of 2 mL. This dilution was layered on 3 mL of Histopaque-1077 [32] in a plastic tube. The samples were centrifuged at 400 × *g* for 30 min at room temperature. The resulting buffy coat of white cells above the erythrocytes was then collected and transferred into a fresh tube. Cells were washed twice with 4 mL of PBS and centrifuged at 400 × *g* for 7 min at room temperature. The final pellet was resuspended in 1 mL of 1% paraformaldehyde (in PBS) to fix the cells. Thirty minutes after resuspension, the cells were centrifuged at 400 × *g* for 7 min at room temperature, the supernatant was discarded, and the cells were washed twice with PBS and centrifuged under the conditions described previously. The final pellet was then resuspended in PBS with 1% BSA [32]. Cells were counted using a Neubauer counting chamber, always by the same person. Single staining was performed using the recommended dilution of the fluorescent primary monoclonal antichicken antibodies [33]. Twenty microliters of this dilution were then mixed with 10^6 mononuclear cells and kept at room temperature in the dark for 20 min. After this incubation period, cells were washed with 2 mL of PBS and centrifuged at 400 × *g* for 7 min at room temperature. The final pellet was resuspended in 250 μL of PBS with 1% BSA. All samples went through cytometry within 2 h of staining.

Monoclonal primary antibodies used in these essays were anti-chicken CD28 [chicken CD28 (40–44 kDa)], clone AV7, IgG1κ; anti-chicken CD8α [α chain of CD8 (34 kDa)], clone CT-8, IgG1κ; mouse anti-chicken monocyte/macrophage, clone KUL01, IgG1κ; anti-chicken TCRαβ [Vβ1; chicken TCR αβ (Vβ1)], clone TCR2, IgG1κ; mouse anti-chicken Bu1 [specific for chicken B lymphocytes (relative molecular mass = 70–75 kDa)].

Flow cytometry was performed on a FACSCalibur flow cytometer [34]. Green fluorescence (from fluorescein isothyocyanate) was detected on the FL1 channel (530/30 nm), and orange fluorescence was detected on the FL2 channel (585/42 nm). Cells were analyzed (minimum of 10,000 events) in the lymphocyte gate, based on forward and side scatter, including contaminating thrombocytes [35]. Data were analyzed with FlowJo software [36].

Statistical Analysis

Each bird was considered as 1 experimental unit for microbiological evaluations, BW, and flow cytometric measurements. *Salmonella* Enteritidis quantification from each sample was

$\log_{10}$ transformed. All data collected were evaluated for normality using the Shapiro Wilk test and for homoscedasticity using Bartlet test. Normal and homoscedastic data were submitted to ANOVA followed by Fisher's LSD post hoc test when treatment effect was detected by ANOVA. A significance level of 0.05 was considered for all tests. When the assumptions of normality and homoscedasticity were not met, a nonparametric Kruskal-Wallis test was performed to assess treatment effects on the evaluated responses, followed by Wilcoxon 2 group test for comparison between treatments. When *Salmonella* Enteritidis quantification was not possible, frequency of positive and negative samples within each treatment were expressed as a percentage and compared using the chi-square test ($P \leq 0.05$). All data, except percentages, are reported as mean ± SD.

RESULTS AND DISCUSSION

Bacterial Shedding and Persistence in the Environment

All samples were collected before birds were negative for *Salmonella*. Cloacal swabs taken 48 h after *Salmonella* infection showed similar concentrations of the pathogen for all challenged groups, indicating that inoculation of 10^5 cfu of *Salmonella* Enteritidis per bird effectively induced dissemination of the pathogen in 100% of the challenged birds. The NC group was *Salmonella* free. Cloacal swabs taken from birds 13 d postinfection (d 28 after hatch) revealed that *Salmonella* shedding was identified in 80% of sampled birds only from the PC group (Table 2). Drag swabs from PC, OAF, and OAW returned positive for *Salmonella* at 21, 28, and 42 d posthatch. The same was observed for OAFW, except at 42 d posthatch, when *Salmonella* was not isolated. The same evaluations of samples from NC group returned negative for *Salmonella* (data not shown).

Bacterial Isolation and Persistence in Crop and Ceca

On d 22 posthatch (7 d postinfection), *Salmonella* was not detected in any sampled organs from the NC group. The bacteria were detected in100% of sampled crop from the PC and OAW groups. This result was significantly higher than that observed in the OAF and OAFW groups. The latter showed the lowest detection rate after the NC group. For ceca samples, the highest *Salmonella* detection rate was observed in samples from the PC group (87.5%), followed by those from OAF (62.5%), OAW (37.5%), and OAFW, in which the bacterium was not detected (0%).

On d 42 posthatch (27 d postinfection), *Salmonella* was only detected in crop and ceca of birds from the PC and OAF groups. Mean bacterial concentration did not significantly differ between these 2 groups for crop samples, whereas ceca samples had a *Salmonella* Enteritidis concentration significantly lower in birds from the OAF group (Table 3). Several studies using different bacterial burdens as well as different means of challenge have shown that birds infected with *Salmonella* and not treated tend to have infection prevalence higher than 40% at slaughter age [12, 22, 37].

The absence of *Salmonella* Enteritidis from swabs of all treated birds on d 28 posthatch (13 d postinfection) indicate that the reduction in pathogen shedding observed on d 22 posthatch may have resulted in lower infective pressure in rooms, which may have led to lower pathogen ingestion by birds, with consequent further lower dissemination. It is important to mention that products were provided only from 1 to 21 and from 35 to 42 d, with this reduction on bacterial shedding happening at 28 d of age, probably or mostly due to factors indirectly related to treatment, as an improvement on the biological status of treated birds and a lower infective pressure in the environment. Borsoi et al. [23], supplying OA and EO to birds, reported a significant reduction in bacterial shedding while birds were being treated with the compounds, and a subsequent increase in shedding when such treatment was withdrawn. Such a condition was not observed in the present study.

Within the OAF and OAFW treatment groups, significantly fewer crop samples were positive for *Salmonella* on d 22 posthatch when compared with PC and OAW. At the same time point, the infection of ceca was most effectively reduced by using the association OAFW, considering the absence of *Salmonella* in ceca of birds from this group. Treatment OAW was the second most effective on d 22 posthatch, fol-

Table 2. Concentration (log_{10} cfu/mL of broth) and incidence (% positive samples) of *Salmonella* Enteritidis in broiler cloaca on d 17 and 28 posthatch (2 and 13 d postinfection, respectively)

Sampling time (d posthatch)	Variable	Treatment[1]				
		NC	PC	OAF	OAW	OAFW
17	*Salmonella* Enteritidis concentration	0 ± 0^{a}	2.94 ± 0.50^{b}	3.40 ± 0.46^{b}	2.44 ± 1.00^{b}	2.74 ± 0.87^{b}
28	*Salmonella* Enteritidis-positive samples	0^{a}	80^{b}	0^{a}	0^{a}	0^{a}

[a,b]Averages (ANOVA followed by Fisher's LSD test) or percentages (chi-squared test) within a row with different superscripts differ significantly ($P < 0.0001$).

[1]NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

Table 3. Concentration (log_{10} cfu/g of content) and incidence (% positive samples) of *Salmonella* Enteritidis in broiler crops and ceca 22 and 42 d posthatch (7 and 27 d postinfection, respectively)

Sampling time	Variable	Treatment[1]				
		NC	PC	OAF	OAW	OAFW
22 d posthatch	*Salmonella* Enteritidis-positive crop	0^{a}	100^{b}	87.5^{b}	100^{b}	25^{a}
	Salmonella Enteritidis-positive ceca	0^{a}	85.7^{d}	62.5^{c}	37.5^{b}	0^{a}
42 d posthatch	*Salmonella* Enteritidis concentration in crop	0.00 ± 0.00^{a}	2.00 ± 1.33^{b}	2.14 ± 1.29^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Salmonella Enteritidis concentration in ceca	0.00 ± 0.00^{a}	1.90 ± 1.40^{b}	0.30 ± 0.90^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[a–d]Averages (Kruskall-Wallis followed by Wilcoxon 2 group test) or percentages (chi-squared test) within a row with different superscripts differ significantly ($P < 0.0001$).

[1]NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

lowed by OAF. Based on these results, the relation between crop positivity versus cecum positivity may be compared among groups. A higher prevalence of the pathogen in crop was noticed in all treatments in which the detection was possible in, at least, one of both sites. This dynamic of *Salmonella* positivity in crop and cecum was most distinct in group OAW (from 100% of positivity in crop to 37.5% of positivity in cecum), whereas in other treatments this reduction was relatively lower (from 100 to 87.5% positivity in PC; from 87.5 to 62.5% positivity in OAF; and from 25 to 0% positivity in OAFW). Results from 5 experiments [38] evaluating the prevalence of positivity in crop and cecum from birds experimentally challenged with *Salmonella* Enteritidis, fasted or not, showed variable relations between positivity in crop and cecum among the assessed groups (sometimes higher prevalence in crop and lower in cecum; sometimes the opposite). Corrier et al. [39], evaluated crop and cecum positivity from fasted birds, without *Salmonella* challenge, and observed lower prevalence in crop than in cecum within the same assessed groups. Similar observations were reported by Byrd et al. [40], who observed, regardless of the treatment, a higher frequency of *Salmonella* Typhimurium occurrence in crop, in relation to cecum occurrence, in experimentally infected birds. Such findings may be due to differences between individuals on the amount of contaminated litter or feed ingested just before the collections, which would directly affect pathogen detection. Thus, higher prevalence in crop (100%) at 22 d in birds from OAW, followed by relative lower prevalence in cecum (37.5%), suggests that part of the crop samples could have a lower pathogen infective concentration; considering that for an effective bacterial colonization, leading to bacterial prevalence in the distal portions of the gastrointestinal tract, factors such as a higher bacterial burden as well as general bacterial conditions are essential [41].

The most accepted antimicrobial effect of OA (short-chain fatty acids) is related to the diffusion of their undissociated form through the semipermeable membrane of the microorganisms into the cell cytoplasm [42–44]. Once inside the cell, where pH is near 7.0, they dissociate and suppress bacterial cell enzymes and nutrient transport systems. This antibacterial action will depend on the pH at which 50% of the acid is dissociated, as well as the chemical form, molecular weight, nature of the microorganism and its related minimum inhibitory concentration value for each acid, the species, and the buffering capacity of the feed [44]. Thus, blends of acids may have broader spectrum of activity. Additionally, as to the antibacterial effect of the acids, both tested products had also OE in its composition, from which carvacrol is the active ingredient. Carvacrol is a phenolic monoterpenoid and a major constituent of oregano. Although related to outer membrane disintegration in gram-negative bacteria [45, 46], the site of action in carvacrol is thought to be the cytoplasmic membrane, resulting in passive transport of ions across the membrane [47]. These mechanisms allied to the antibacterial action of the acids may offer synergism regarding antibacterial effect of both tested products.

Results at slaughter age (42 d posthatch) indicate that supplying blends of OA and OE through the water (OAW) and combining drinking water and feed supply (OAFW) led to the absence of *Salmonella* in crop and in cecum of all treated birds, whereas supplying only the feed (OAF) led to a significantly reduction of the pathogen in cecum. The presence of *Salmonella* in crop at rates similar to the PC for the OAF group at this mentioned point may be due to a prior lower reduction (on d 22 posthatch) on the pathogen burden, although such a reduction was significant at that time point.

Flow Cytometric Evaluations

The peripheral blood subsets of immune cells showed trends that may explain the outcomes observed during the experiment. The percentage of circulating macrophages was similar between groups on d 14 posthatch (1 d before infection), but within 7 d postinfection the number of these cells had decreased in the PC group in relation to the NC ($P = 0.09$; Figure 1). At slaughter age, the proportions of all groups were again similar (data not shown). A trend for reduction in the proportion of $CD4^{-}TCRV\beta1^{+}$ cells was also noticed on d 7 postinfection between the NC and all the infected groups; additionally, the amount of $CD8^{+}CD28^{-}$ cells showed a visual increase in relation to control in all infected groups apart

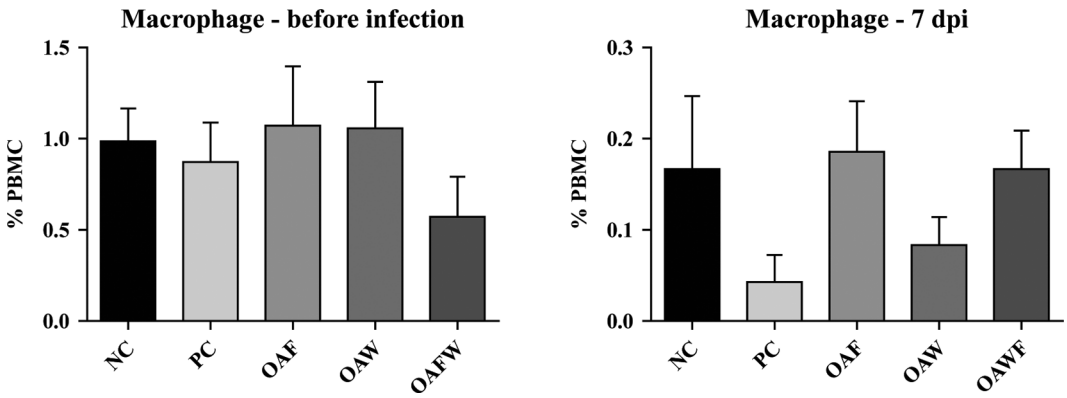

Figure 1. Percentages of circulating macrophages. The y-axis presents the percentages of positive cells in relation to the peripheral blood mononuclear cells (PBMC). NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water; dpi = days postinfection.

from OAW (Figure 2). Even though these results are not statistically significant, they are relevant because these outcomes have been described in literature. It is known that at 7 d following a *Salmonella* infection a marked increase in the amount of $CD8^+CD28^-$ circulating cells and a reduction in numbers of $TCRV\beta1^+$ cells occurs [31, 48, 49]. The variation on the number of mononuclear circulating cells is expected to derive from the migration of cells of both arms of the immune system as a consequence of infection and as a response to the chemotactic signals produced by macrophages, which are among the cells in the first line of defense. In the beginning of the infection, the exit of cells from the circulation is superior to the regenerative capacity of the primary lymphoid organs [50–52]. Lymphocytes quickly enter the infection site, of which the main phenotype is $TCRV\beta1^+$ [53]. The number of $CD8^+CD28^-$ is expected to be increased, as this subset is quickly produced in response to acute infection, and does not exist before such stimulus [54]. Because the number of macrophages is reduced but it is not followed in the same proportions by the lymphocytes, it indicates that the response has not yet fully activated the adaptive arm.

The CD4-to-CD8 ratio has been associated with immunocompetence [55]. In the present work, the OAW group had a significantly elevated ratio at 7 d postinfection compared with all other groups ($P < 0.05$). This ratio is stable under various infectious conditions at 7 d postinfection, and therefore the increased ratio may indicate that a significant interaction occurred with the administered supplement and the infectious challenge.

At the slaughter age (27 d postinfection), the most relevant observation was a reduction in the proportion of $CD8^+CD28^+$ lymphocytes in all infected groups, being statistically significant between OAW and the NC (Figure 3). This subset of cells is expected to be reduced after a *Salmonella* infection [31]. The CD8 cells are crucial to avoid bacterial translocation from or between mucosal sites [56]. The presence of $CD8^+CD28^+$ cells in virus-infected tissues, and therefore in a lower concentration in the circulation, has been associated with a positive clinical outcome in humans [54, 57, 58] and could help explain the good microbiological results of the OAW group, for instance.

Performance Variables

Mean BW were affected by treatments on d 21 posthatch (6 d postinfection), when the PC and NC groups showed significant differences, and on d 35 posthatch. At this point, OAW and OAFW showed higher BW means compared with NC. No other differences were found for this variable. As total feed consumption was

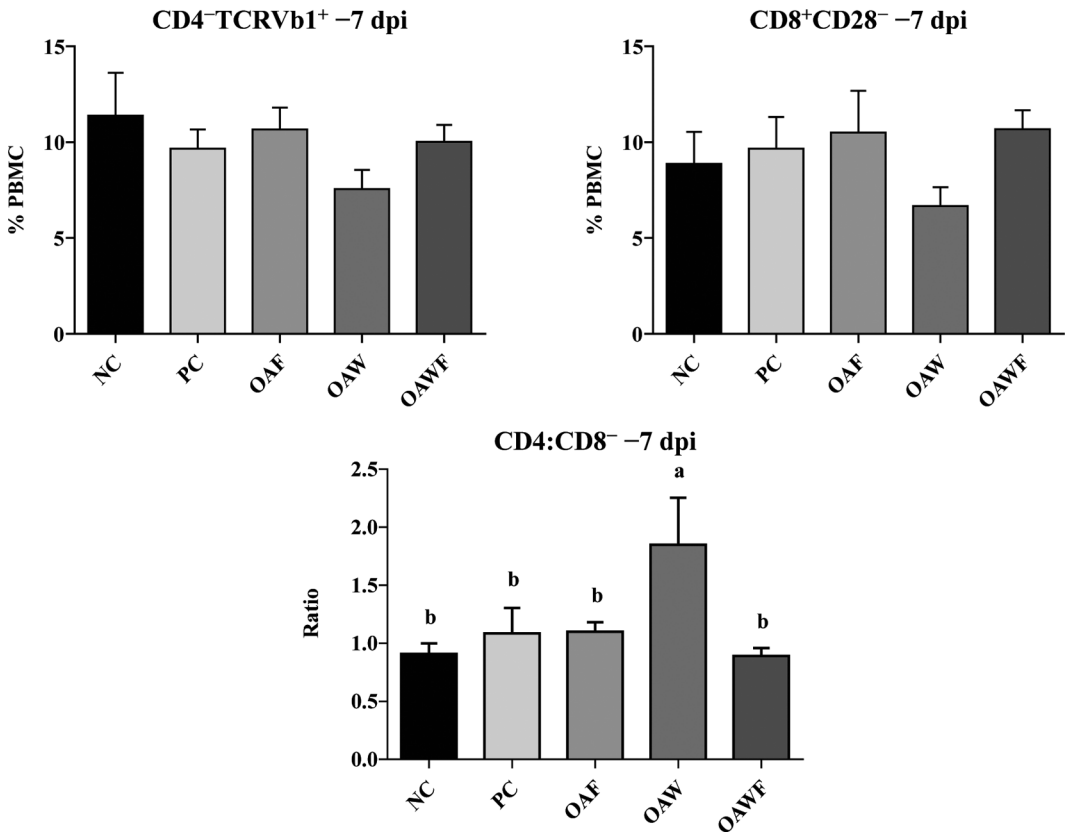

Figure 2. Percentages of circulating lymphocyte subsets at 7 d postinfection (dpi) and the ratio between cluster of differentiation (CD) 4^+ and $CD8^+$ cells. The y-axis presents the percentages of positive cells in relation to the peripheral blood mononuclear cells (PBMC) or the ratio between the positive subsets, in the case of CD4:CD8. Different letters (a,b) indicate statistically significant differences ($P < 0.05$) in a Bonferroni test. NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

quantified per room, only 1 value was available per group, precluding any meaningful comparison between treatment results. Additionally, comparisons of feed consumption and FCR between treatments were not within the scope of this trial. Values obtained for FCR in the whole experimental period ranged from 1.68 to 1.80 among treatments and were considered normal (data not shown).

The challenge model was not only demonstrated to be sufficient related to pathogen dissemination; it was also shown to have a detrimental effect on BW (Table 4). Twenty-two days posthatch (7 d postinfection), mean BW of the PC group was significantly lower compared with the NC group. None of the other challenged and treated groups were affected in a similar way. Yan et al. [59] also reported a BW reduction in birds challenged with 10^8 cfu of *Salmonella* Enteritidis at 5 d after infection. Marcq et al. [60], evaluating challenges with different *Salmonella* Typhimurium concentrations, reported a decrease of about 14.5% in BW gain from challenged birds, within the postinfection periods, compared with a nonchallenged control. It has been reported that livestock performance is decreased when challenged by different pathogens due to an increase in energetic and proteic requirements, which may vary according to the type of pathogen and the pathogenic burden. Such increased requirements are a result of building an immune response, repairing or re-

Table 4. Mean BW (g/bird) at different time points along the trial

	Treatment[1]				
Age (d)	NC	PC	OAF	OAW	OAFW
14	474.7 ± 37.5	439.8 ± 50.2	459.1 ± 29.8	441.6 ± 48.5	429.3 ± 38.3
21	923.4 ± 66.5[a]	856.4 ± 89.4[b]	883.5 ± 41.1[ab]	903.1 ± 60.2[ab]	894.4 ± 51.6[ab]
35	1,855.2 ± 206.1[B]	1,963.3 ± 318.8[AB]	2,059.3 ± 131.1[AB]	2,173.4 ± 140.1[A]	2,182.40 ± 111.89[A]
42	2,729.90 ± 280.92	2,754.0 ± 341.30	2,781.8 ± 204.72	2,901.9 ± 209.64	2,942.2 ± 166.68

[a,b]Lowercase letters (ANOVA followed by Fisher's LSD test) within a row differ significantly ($P < 0.05$).

[A,B]Uppercase letters within a row differ significantly ($P < 0.01$).

[1]NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

placing damaged tissues and fluids, and also the onset of fever [60].

CONCLUSIONS AND APPLICATIONS

1. Challenging broilers at 15 d of age with 10^5 cfu/bird of *Salmonella* Enteritidis was sufficient to induce a prevalent infection until slaughter age.

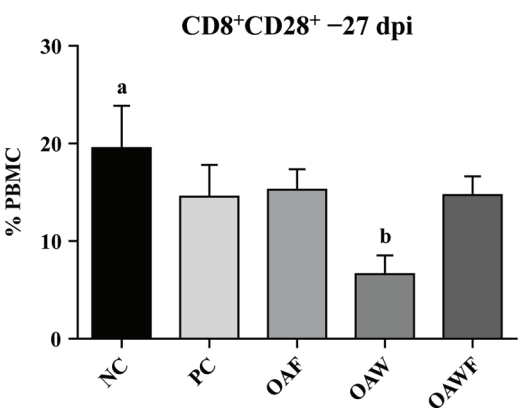

Figure 3. Percentages of circulating cluster of differentiation (CD) 8^+CD28^+ lymphocytes at 27 d postinfection (dpi). The y-axis presents the percentages of positive cells in relation to the peripheral blood mononuclear cells (PBMC). Different letters (a,b) indicate statistically significant differences ($P < 0.05$) in an ANOVA and Bonferroni post-test. NC = negative control (not challenged with *Salmonella* Enteritidis, not treated); PC = positive control (challenged with 10^5 cfu of *Salmonella* Enteritidis; not treated); OAF = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the feed; OAW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acids and oregano extract through the water; OAFW = challenged with 10^5 cfu of *Salmonella* Enteritidis; treated with organic acid and oregano extract through the feed and water.

2. Supplying water with OA and OE at 0.08%, as well as supplying both feed at 0.2% and drinking water at 0.08% with evaluated blends, both during the 3 first weeks of life of birds and 1 wk before slaughter, was effective in significantly reducing the prevalence of infection in birds at 22 d of age, as well as in eliminating the pathogen shedding at 42 d of age.
3. Supplying only feed with OA and OE at 0.2% during the study periods was effective to significantly reduce the pathogen shedding at 22 and 42 d of life, but not in reducing *Salmonella* Enteritidis prevalence in crop at the slaughter age.
4. Results suggest that applying OA and OE to both feed and water may be effective on-farm tools to be used for the control of *Salmonella* Enteritidis persistence during the broiler chicken production cycle.

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