Postprint: Control Efficacy of Different Essential Oil and Acidifier Combinations against Salmonella enteritidis Infection in Broiler Chickens

Authors: Zhao Jingpeng, Peiyong Li, Wang Hongyu, SONG Yizhen, Sun Shuhong, forest

Date: 2018-12-24T00:00:00+00:00

Abstract

This experiment aimed to investigate the control effects of different combinations of essential oils and acidifiers on *Salmonella enteritidis* infection in broiler chickens. A total of 840 1-day-old Arbor Acres broiler chickens, free of *Salmonella enteritidis*, were randomly allocated into 7 groups and housed in 7 structurally identical but mutually isolated rooms, with 6 replicates per group and 20 chickens per replicate (half male and half female). Group 1 (control group) was fed a basal diet, Group 2 (antibiotic group) was fed an experimental diet supplemented with 40 g/t enrofloxacin hydrochloride in the basal diet, and Groups 3–7 (essential oil and acidifier groups) were fed experimental diets supplemented with 800 g/t of different combinations of organic acids and essential oils (Formulations A, B, C, D, E) in the basal diet. At 14 days of age, each chicken was orally administered 200 L of SDBL-1 *Salmonella enteritidis* culture (5$×10^{7}$ CFU/mL). At 2, 7, and 14 days post-challenge (i.e., at 16, 21, and 28 days of age), 2 chickens per replicate were randomly selected for blood collection, slaughter, and sampling. The experimental period was 28 days, divided into two feeding phases: 1–21 days of age and 22–28 days of age. The results showed: 1) Both pre- and post-challenge, compared with the control group, Formulation A significantly reduced the feed conversion ratio of broiler chickens ($P<0.05$), while Formulations D and E improved performance only post-challenge and pre-challenge, respectively ($P<0.05$). 2) Based on tissue bacterial load, Formulations A and D exhibited good control effects against *Salmonella enteritidis* infection in broiler chickens. 3) Following *Salmonella enteritidis* infection, compared with the control group, the formulations significantly reduced serum tumor necrosis factor-α (TNF-α) content at 28 days of age ($P<0.05$) and significantly increased serum immunoglobulin G (IgG) content at 16 days of

age (P<0.05), while Formulation E showed the opposite effects. These findings indicate that both Formulations A and D can inhibit *Salmonella enteritidis* in broiler chickens, with Formulation A being suitable for long-term feeding, while Formulation D is recommended only during infection status.

Full Text

Effects of Different Combinations of Essential Oil and Organic Acid on Salmonella enteritidis Infection in Broiler Chickens

ZHAO Jingpeng[1], LI Peiyong[1]*, WANG Hongyu[2], SONG Yizhen[2], SUN Shuhong[1], LIN Hai[1]

[1]Shandong Key Laboratory of Animal Biotechnology and Disease Control, College of Animal Science and Technology, Shandong Agricultural University, Tai'an 271018, China
[2]Shanghai Menon Biotechnology Co., Ltd., Shanghai 201807, China

Abstract

This study investigated the control effects of different essential oil and organic acid combinations on Salmonella enteritidis (SE) infection in broiler chickens. A total of 840 one-day-old Arbor Acres broiler chicks, confirmed negative for SE infection, were randomly allocated into 7 groups and housed in 7 separate but identically configured rooms, with 6 replicates per group and 20 birds per replicate (half male, half female). Group 1 (control) received a basal diet, group 2 (antibiotic group) received the basal diet supplemented with 40 g/t enrofloxacin HCl, and groups 3–7 (essential oil and acidifier groups) received the basal diet supplemented with 800 g/t of different organic acid and essential oil combinations (formulations A, B, C, D, and E). At 14 days of age, each bird was orally challenged with 200 L of SDBL-1 SE suspension (5$×10^{7}$ CFU/mL). At 2, 7, and 14 days post-challenge (16, 21, and 28 days of age), two birds per replicate were randomly selected for blood collection, slaughter, and tissue sampling. The 28-day experiment consisted of two feeding phases: days 1–21 and days 22–28. The results showed: (1) Compared with the control group, formulation A significantly reduced the feed-to-gain ratio (F/G) of broilers both before and after challenge (P<0.05), while formulations D and E improved performance only in the post- and pre-challenge periods, respectively (P<0.05). (2) Based on tissue bacterial load, formulations A and D demonstrated good control effects against SE infection. (3) Following SE infection, formulation A significantly reduced serum tumor necrosis factor-α (TNF-α) content at 28 days of age (P<0.05) and significantly increased serum immunoglobulin G (IgG) content at 16 days of age (P<0.05), whereas formulation E showed the opposite effects. These findings indicate that both formulations A and D can inhibit SE in broiler chickens, with formulation A being suitable for long-term feeding and formulation D recommended only during infection status.

Keywords: essential oil; organic acid; Salmonella enteritidis; bacterial load; broiler chickens

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Salmonellosis, caused by Salmonella, is a major zoonotic and foodborne disease, with poultry considered the primary reservoir. Salmonella can vertically infect chicks through contaminated eggs from infected breeder hens or spread horizontally within flocks via feces, contaminants, or vectors through the digestive and respiratory tracts. While the implementation of the U.S. National Poultry Improvement Plan (NPIP) has effectively controlled two Salmonella serotypes —pullorum disease and fowl typhoid—in imported American breeder stock in China, these diseases still occur in local breeds or domestically developed strains. Currently, avian paratyphoid-Salmonella enteritidis poses a major threat to egg and broiler flocks in China, causing reduced production performance and diminished meat and egg quality that result in significant economic losses, while also causing human food poisoning incidents through poultry products and creating public health concerns.

During Salmonella control, antibiotics and vaccines have played important roles. However, the irrational use of antibiotics has led to widespread antimicrobial resistance, while vaccine protection is limited to homologous or same-serotype Salmonella infections and interferes with flock eradication programs. Therefore, there is an urgent need to identify green, efficient anti-Salmonella products suitable for poultry production. Both in vivo and in vitro studies have demonstrated that organic acids (such as benzoic acid and medium-short chain fatty acids) and plant extracts (such as essential oils including cinnamaldehyde, carvacrol, eugenol, and thymol) exhibit strong inhibitory effects against Salmonella, with synergistic efficacy between the two. When present in their undissociated molecular form, organic acids can penetrate bacterial cell walls and disrupt the normal physiology of pH-sensitive bacteria, while essential oils can damage bacterial cell membranes, facilitating organic acid entry into the cytoplasm. To prevent absorption in the upper digestive tract, particularly the crop, poultry organic acid or essential oil products are often coated to enable slow release throughout the gastrointestinal tract, ensuring their regulatory effects on microecological balance in the distal small intestine. The combined effects of organic acids and essential oils depend not only on their individual antimicrobial activities and compatibility but also on complex interactions with coating technologies. Therefore, scientific screening of individual components for compounding is crucial. This study designed five formulations of organic acids and natural plant essential oils, processed them with sustained-release technology, and comparatively evaluated their effects against SE infection in broiler chickens to provide a basis for future development and application of such products.

1.1 Experimental Materials

The essential oil and organic acid formulations were provided by Shanghai Menon Biotechnology Co., Ltd. and produced using self-emulsifying sustained-release solid dispersion technology. The SE strain was isolated and identified by our laboratory in 2015 from broiler cases in Tai' an, Shandong Province, and designated SDBL-1. Enrofloxacin HCl was purchased from Zhejiang Guobang Pharmaceutical Co., Ltd., with an active ingredient content of 98%.

1.2 Experimental Design and Management

Nine hundred 1-day-old Arbor Acres broiler chicks were purchased from a local hatchery. Cloacal swabs were collected to detect SE infection, and 840 SE-negative individuals with similar body weight were selected and randomly divided into 7 groups. Each group was housed in one of 7 structurally identical but isolated rooms, with 6 replicates per group and 20 birds per replicate (half male, half female). Birds were raised in tiered cages (3 tiers, 2 replicates per tier), with 2 cages (1.48 m × 0.68 m) constituting one replicate and 10 birds per cage. Group 1 (control) received the basal diet (Table 1), group 2 (antibiotic group) received the basal diet supplemented with 40 g/t enrofloxacin HCl, and groups 3–7 (essential oil and acidifier groups) received the basal diet supplemented with 800 g/t of different organic acid and essential oil combinations (Table 2). At 14 days of age, each bird was orally inoculated with 200 L of SDBL-1 SE suspension (5$×10^{7}$ CFU/mL). At 2, 7, and 14 days post-challenge (16, 21, and 28 days of age), two birds per replicate (one per cage, 12 per group) were randomly selected for blood collection, slaughter, and tissue sampling. The 28-day experiment included two feeding phases: days 1–21 and days 22–28. Birds had ad libitum access to feed and water throughout the experiment. Temperature, humidity, and lighting were controlled uniformly across all rooms to meet the environmental requirements of white-feathered broilers.

1.3.1 Production Performance

Before challenge (day 14) and at the end of the experiment (day 28), birds in each replicate were fasted and weighed, and feed consumption was recorded to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G). Mortality and culling numbers were also recorded.

1.3.2 Internal Organ Indices

At 9, 16, 21, and 28 days of age, two birds per replicate (one per cage, 12 per group) were randomly selected, weighed, and slaughtered. The liver, spleen, and bursa of Fabricius were collected and weighed individually to calculate organ indices (100 × organ weight/body weight, %).

1.3.3 Tissue Bacterial Load

At 16, 21, and 28 days of age, two birds per replicate (one per cage, 12 per group) were randomly selected, weighed, and slaughtered. Appropriate amounts of liver, spleen, and cecal contents were collected, mixed with 3 mL sterile phosphate-buffered saline (PBS), homogenized in an ice bath for 2 min, and subjected to 10-fold serial dilutions. Aliquots of 100 L of the original solution and each dilution were spread evenly on XLT-4 agar plates and incubated at 37°C for 24 h in a biochemical incubator. After incubation, five suspicious colonies were selected and identified through biochemical and serological tests to calculate SE load.

1.3.4 Serum Biochemical Indices

At 16, 21, and 28 days of age, two birds per replicate (one per cage, 12 per group) were randomly selected, and 2 mL of blood was collected from the brachial vein. Serum was separated and stored at -20°C. Serum tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), immunoglobulin G (IgG), and secretory immunoglobulin A (SIgA) contents were determined using enzyme-linked immunosorbent assay (ELISA) kits purchased from Jiangsu Yutong Biotechnology Co., Ltd.

1.4 Statistical Analysis

Experimental data were analyzed using the GLM procedure in SAS 9.0 software for one-way ANOVA. Means were compared using Tukey' s HSD method, with $P<0.05$ indicating significant differences.

2.1 Production Performance

As shown in Table 3 , under normal rearing conditions (pre-challenge), all formulations except D and enrofloxacin HCl significantly reduced the F/G of broilers during days 1-14 compared with the control group ($P<0.05$), with formulation A showing the best effect. This improvement primarily resulted from increased ADG ($P<0.05$) rather than changes in ADFI ($P>0.05$), suggesting a growth-promoting effect of formulation A. Following SE infection, all formulations except E significantly increased ADG and decreased F/G during days 15-28 ($P<0.05$), while formulation E significantly reduced ADFI ($P<0.05$) without improving performance. Formulations A and D showed relatively better effects. Over the entire experimental period (days 1-28), enrofloxacin HCl and formulations A, B, and D significantly increased ADG compared with the control group ($P<0.05$), while enrofloxacin HCl and formulations A and D also significantly increased ADFI ($P<0.05$), except for formulation B.

2.2 Internal Organ Indices

As shown in Table 4 , under normal rearing conditions (pre-challenge), enrofloxacin HCl and formulation B significantly reduced liver index at 9 days

of age ($P<0.05$), while enrofloxacin HCl and formulations B, C, and E significantly reduced spleen index at 9 days ($P<0.05$). However, after SE infection, the inhibitory effects of these treatments on organ development diminished. Only enrofloxacin HCl and formulation E reduced liver index at 16 and 21 days of age, respectively ($P<0.05$). At 28 days of age, formulation C showed higher spleen index than formulation B ($P<0.05$).

2.3 Tissue Bacterial Load

As shown in Table 5 , at 2 days post-challenge (16 days of age), enrofloxacin HCl and all formulations significantly reduced liver bacterial load ($P<0.05$), but formulation E significantly increased spleen bacterial load ($P<0.05$). At 7 days post-challenge (21 days of age), all formulations except enrofloxacin HCl and formulations B and D (for liver) and formulation E (for cecal content) showed inhibitory effects. At 14 days post-challenge (28 days of age), enrofloxacin HCl and all formulations significantly reduced cecal content bacterial load ($P<0.05$), with formulations A and D showing superior inhibitory effects on spleen bacterial load compared with enrofloxacin HCl ($P<0.05$).

2.4 Serum Inflammatory Factors and Immunoglobulin Content

As shown in Table 6 , regarding serum inflammatory factors, formulations A and D significantly reduced serum TNF-α content at 28 days of age ($P<0.05$), formulation D significantly reduced serum IL-6 content at 16 days ($P<0.05$), while formulation E significantly increased serum TNF-α at 21 days and IL-6 at 21 and 28 days ($P<0.05$). For serum immunoglobulins, formulation A significantly increased serum IgG content at 16 days ($P<0.05$), whereas formulations C and E significantly reduced IgG content at 21 and 28 days, respectively ($P<0.05$).

3 Discussion

We selected the SDBL-1 SE strain for challenge to: (1) differentiate the source of Salmonella isolated from organs, determining whether SE originated from inoculation or the environment; and (2) inoculate a single serotype to reduce interference from multiple serotypes. To prevent cross-infection of the challenge strain among groups and ensure result reliability, birds were housed in seven isolated rooms with identical construction, layout, rearing methods, and management to maintain uniform environmental conditions, consistent with randomized block design requirements. Before challenge, broilers were pre-fed antibiotics or different organic acid and essential oil formulations for 2 weeks to allow gastrointestinal adaptation to dietary treatments. Control group birds each received 10^7 CFU of SE, resulting in substantial SE colonization in cecal contents, liver, and spleen, with cecal loads substantially higher than hepatic or splenic loads. This indicates that SE preferentially colonizes the cecum before infecting other organs, confirming successful establishment of the challenge model.

Both organic acids and natural plant essential oils comprise many types with varying antimicrobial efficacies. While numerous studies have examined the effects of different organic acid or essential oil complexes on Salmonella infection in broilers, few have considered their additive or interactive effects, particularly under sustained-release conditions. Based on tissue bacterial load, our study found that four of the five tested essential oil and organic acid formulations showed stronger inhibitory effects against SE than enrofloxacin HCl, with formulation A providing the best protection, followed by formulation D. Compositionally, formulations A, B, and C all contained carvacrol, thymol, and benzoic acid; formulations A and D both contained benzoic and butyric acids; while formulations B, C, and D all contained benzoic acid. We therefore speculate that butyric acid exhibits synergistic antimicrobial effects with other essential oil components against SE. Butyric acid is a short-chain fatty acid belonging to the monocarboxylic acid group, along with formic, acetic, and propionic acids. This inference aligns with Zhou et al., who reported that thymol combined with acetic acid showed superior bactericidal effects against Salmonella typhimurium compared with individual supplementation. Although formulation E contained the same active components as formulation A, the individual ingredient levels were 1.5 times higher in formulation E, yet resistance to SE decreased rather than increased, suggesting that the combined effects of organic acids and essential oils depend on rational dosage matching. While we cannot completely exclude that the antimicrobial properties of formulations A and D resulted solely from butyric acid, as butyric acid itself possesses strong anti-SE activity, particularly when coated, this possibility seems unlikely given that previous studies observed beneficial effects of butyric acid at doses (1‰) far exceeding those in formulation E (0.012‰).

As a Gram-negative bacterium, SE may trigger inflammatory responses through antigenic molecules such as lipopolysaccharide (LPS) and endotoxins, stimulating macrophage secretion of inflammatory cytokines and inducing immune responses including antibody synthesis by plasma or effector B cells. Our study found that post-infection, formulation A reduced peripheral blood TNF-α and IL-6 release while increasing IgG content, with effects superior to enrofloxacin HCl, whereas formulation E produced opposite effects. These findings correspond with organ bacterial load results, demonstrating formulation A' s beneficial effects on humoral immunity. Combined with internal organ development data, we conclude that formulation E provides minimal control against SE infection. Since no significant differences were observed in serum SIgA content among groups, organic acid and essential oil combinations may not affect mucosal immunity.

Beyond antimicrobial activity, organic acids and essential oils participate in nutrient metabolism. Organic acids reduce dietary pH and promote intestinal digestion, while essential oils increase bile salt secretion and enhance enzyme activity in intestinal mucosa and pancreas. These beneficial effects ultimately reflect in production performance. Studies have shown both positive effects of essential oil and organic acid combinations on broiler feed conversion and absence of

synergistic effects, depending on formulation composition. In our study, formulation A improved ADG and feed utilization both pre- and post-challenge, while formulation D was effective only post-challenge, suggesting that the combination of butyric acid with carvacrol and thymol possesses both growth-promoting and antimicrobial dual functions, whereas butyric acid combined with cinnamaldehyde exhibits only antimicrobial activity. Therefore, formulation A is suitable for long-term feeding, while formulation D is recommended only during infection status. Notably, formulation E promoted growth pre-challenge but lost all efficacy post-challenge, further demonstrating that the ratio of organic acids to essential oils influences combined antimicrobial capacity.

4 Conclusion

First, regarding antimicrobial activity alone, both formulations A and D exhibit good inhibitory effects against SE in broiler chickens, suggesting that the synergistic effect of butyric acid with other essential oil components in coated form is dose-dependent. Second, considering production performance, formulation A is suitable for long-term feeding, while formulation D is recommended only during infection status.

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Note: Figure translations are in progress. See original paper for figures.