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Efficacy of Eugenol Against a *Salmonella enterica* serovar Enteritidis Experimental Infection in Commercial Layers in Production

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Primary Audience: Veterinarians, Researchers, Nutritionists, Poultry Managers

SUMMARY

An experimental study (15-wk-old ISA Brown pullets) was conducted to establish the efficacy of the essential oil of *Eugenia caryophyllata* against *Salmonella enterica* serovar Enteritidis. The trial was composed of 4 groups. Pullets in groups 3 and 4 were fed with a commercial compound feed, and pullets in groups 1 and 2 were fed with the same feed plus the aromatic product at the dose of 250 g/Tm. At 19 wk old, the pullets in groups 1 and 3 were infected individually with an inoculum of $3.2 \pm 0.8 \times 10^7$ cfu of *Salmonella enterica* serovar Enteritidis/pullet. During the postinoculation period, samples of feces and eggs were cultured, and pullets were killed 30 d postinoculation. The aromatic product containing eugenol seems to aid in the cleaning of the intestinal and systemic infections, and it also plays an important role in the control of *Salmonella* cross contamination in eggs.

Key words: eugenol, *Eugenia caryophyllata*, *Salmonella enterica* serovar Enteritidis, laying hen

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DESCRIPTION OF PROBLEM

For years, foodborne illnesses resulting from consumption of food contaminated with pathogenic bacteria, their toxins, or both have been of vital concern to public health [1]. Aromatic plants have been used since ancient times for their preservative and medicinal properties and to impart aroma and flavor to food. The pharmaceutical properties of these medicinal plants and spices are partially attributed to essential oils [2, 3].

Antimicrobial agents, including food preservatives, organic acids, and spices, have been used to inhibit foodborne bacteria and extend the shelf life of processed food [4]. Many naturally occurring compounds found in edible and medicinal plants, herbs, and spices have been shown to possess antimicrobial functions and could serve as a source of antimicrobial agents against food pathogens [5–7]. The phenolic compounds from the essential oils possess a high antimicrobial activity in vitro against foodborne pathogens.

Antibiotic growth-promoters (**AGP**) have been used for the control of pathogens such as *Escherichia coli* or *Clostridium perfringens* (that can have an influence on digestibility), or *Salmonella* (which, furthermore, is also responsible for zoonosis). Prebiotics, probiotics, or

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competitive exclusion mechanisms have been considered as a way of controlling the digestive tract microflora to make the proliferation of pathogens more difficult and favor the growth of beneficial microorganisms such as *Lactobacillus* [8].

Recently, the restrictions on the use of antibiotics as animal growth-promoters have encouraged the use of essential oils as alternatives to AGP. The metabolic properties of essential oils have been shown to increase digestibility and absorption of the nutrients by 5% and increase weight of the newborn litter by up to 18% [9]. In our field studies using an aromatic product composed of clove essential oil from a natural source as a natural alternative to AGP, this aromatic product has surprisingly shown a capacity to control *Salmonella* in eggs. This led to a study to observe the activity of clove essential oil in a controlled infection with *Salmonella enterica* serovar Enteritidis on an experimental farm [10] based on the studies of Humphrey et al. [11, 12], Aabo et al. [13], Berchieri et al. [14], Nakamura et al. [15], and Wigley et al. [16].

MATERIALS AND METHODS

Experimental Design

In the trial, 100 commercial 15-wk-old ISA Brown laying pullets were obtained from a flock [17] that had tested negatively for *Salmonella* in the feces 2 wk before moving them to the experimental farm in Reus (Tarragona), Spain (Centre de Sanitat Avícola de Catalunya i Aragó). The pullets were vaccinated following the standard program for commercial laying hens (excluding the vaccine against *Salmonella* spp.).

At the trial farm, the pullets were kept under observation and acclimatized for 9 d. After this period, they were distributed into 4 groups, with pullets in groups 1 and 2 fed on a daily basis during the whole length of the study, a commercial feed with the aromatic product containing eugenol [18] at the dose of 250 ppm. Pullets in group 3 and 4 were fed with the same feed without the aromatic product. None of the hens received any other medication than the one of its treatment.

The pullets were kept in separated wire cages, identified with specific numeric codes. According to the Directive 86/609/EEC and in application of the Decret 214/1997/DOGC regarding the regulation of animals destined for scientific experimentation, the hens were kept in the final pens with a minimal space of 550 cm^2/ pullet.

Table 1. Detailed description of the experimental design of the study

Group	Hens, n	Aromatic product (250 ppm)	Inoculated with *Salmonella* Enteritidis (3.2×10^7 cfu/mL)
1	1 × 25	Yes	Yes
2	1 × 25	Yes	No
3	1 × 25	No	Yes
4	1 × 25	No	No

After 3 wk on the experimental farm consuming feed added with a commercial product containing eugenol [18], the animals in group 1 (treated with essential oil) and group 3 (positive control) were inoculated with *Salmonella* Enteritidis. Inoculation into the crop was performed individually at a single dose of 1 mL using a syringe containing approximately 3.2×10^7 cfu *Salmonella* Enteritidis. Groups 2 (treated with essential oil) and 4 (negative control) were not inoculated (Table 1).

During the postinoculation period, samples of feces and eggs were taken. Hens were slaughtered at 30 d postinoculation.

During the trial, water and food were supplied ad libitum. The drinking water given to the animals came directly from the mains. A 24-h lighting program was established. The sanitary condition of the feed was controlled and certified previously by the compound feed supplier to ensure the absence of *Salmonella* spp. In addition, the weight, feed consumption, and rate of lay of the hen were monitored during the trial.

Salmonella Culture

Salmonella Enteritidis was isolated using the ISO 6579:2002 test. This modified test is based on Rappaport-Vassiliadis semisolid media for selective *Salmonella* spp. from other bacteria in dust and feces samples. The detection of *Salmonella* necessitates 4 successive stages: preenrichment in nonselective liquid medium (buffered peptone water at 37 ± 1°C for 18 h ± 2 h), enrichment on selective semisolid medium (modified semisolid Rappaport-Vassiliadis agar plates incubated at 41.5 ± 1°C for 24 h ± 3 h; if

Table 2. *Salmonella* Enteritidis isolations from feces, eggs, tested tissues, and serum antibodies detection in groups 1, 2, 3, and 4[1]

	Salmonella Enteritidis isolations															
	Feces[2]				Eggs[3]				Tissues tested[4]				Serum antibodies detection[5]			
Age, wk (days postinoculation)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
15 wk (0 d)	−	−	−	−												
17 wk (0 d)					−	−	−	−					0/23	0/23	1/23	0/23
19 wk (0 d)	−	−	−	−	−	−	−	−					0/25	0/25	2/25	1/25
20 wk (1 d)	+	−	+	+												
21 wk (4 d)	+	−	+	−	+	−	+	−								
22 wk (12 d)	+	−	+	−	+	−	+	+								
22 wk (15 d)									+	−	+	−	3/5	0/5	4/5	0/5
23 wk (19 d)	+	−	+	−	+	−	+	+								
24 wk (30 d)					−	−	+	−	−	−	+	−	4/5	0/5	5/5	0/5

[1]Groups: 1 = treated and inoculated; 2 = treated and not inoculated; 3 = not treated and inoculated (positive control); 4 = not treated and not inoculated (negative control).

[2]Feces = 25 g/pen was analyzed.

[3]Eggs = number of eggs analyzed. All eggs laid for each period were pooled and analyzed.

[4]Tissues tested = overview of *Salmonella* Enteritidis isolation in internal organs (see Table 3).

[5]Serum antibodies detection = serologic results by ELISA.

a plate is negative after 24 h, it is incubated for further 24 ± 3 h), selective plating and identification (2 selective solid media are inoculated: xylose-lysine-deoxycholate agar and Difco Brilliant Green agar [19], incubated at 37 ± 1°C and examined after 24 ± 3 h), and confirmation of identity stage (colonies of presumptive *Salmonella* are subcultured, then plated out, and their identity is confirmed by means of Triple Sugar Iron [20] and BBL Crystal Identification System Kit [21]). An antibody microarray assay was developed for *Salmonella* serotyping based on the Kauffmann-White scheme. Serum was analyzed for antibody against *Salmonella* using a commercial ELISA kit.

Aromatic Product

The aromatic product [18] was composed of essential oil from a natural source of *Eugenia caryophyllata* or *Syzygium aromaticum* [22], in an amorphous SiO_2 inert carrier. The concentration of eugenol in the aromatic product was 20%. The components of the essential oil were chemically valuated through gas chromatography-quadrupole mass spectroscopy [23, 24]. It was observed that its profile is as described in the flavoring substances classifications [3, 25, 26] regarding *E. caryophyllata*.

Inoculum Preparation

The inoculum was developed in the Centre de Sanitat Avícola de Catalunya i Aragó from a field strain of *Salmonella enterica* serovar Enteritidis isolated from a naturally infected laying flock. The strain was kept in a pure culture and grown in a nutrient broth [27] at 37°C for 24 h. The microorganism count was carried out at a dilution of 10 bases in phosphate buffer [28] and plated onto solid medium Brilliant Green agar [19]. From the obtained count, the liquid medium was diluted to a concentration of 10^8 *Salmonella* Enteritidis/mL.

The inoculum solution was prepared 24 h before it was given to the hens, with a count of 8.5 ± 0.15 × 10^7 cfu *Salmonella* Enteritidis/mL. Another recount was carried out when the inoculum was given to the hens, with the result of 3.2 ± 0.08 × 10^7 cfu *Salmonella* Enteritidis/mL.

Collection of Samples

Fresh Feces. A 25-g sample of fresh feces per pen was collected from the bottom of the cage. Samples were analyzed collectively for each group 2 wk before the inoculation, 1 d before the inoculation, and after the challenge overnight feces were collected and analyzed weekly.

Table 3. *Salmonella* Enteritidis isolation in viscera in groups 1, 2, 3, and 4[1]

	Salmonella Enteritidis isolation in viscera															
	Ceca				Liver + spleen				Ovary				Organs pool[2]			
Age, wk (days postinoculation)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
22 wk (15 d)[3]	4/5	0/5	4/5	0/5	5/5	0/5	3/5	0/5	1/5	0/5	0/5	0/5				
24 wk (30 d)[4]	–	–	+	–									–	–	+	–

[1]Groups: 1 = treated and inoculated; 2 = treated and not inoculated; 3 = not treated and inoculated (positive control); 4 = not treated and not inoculated (negative control).

[2]Organs pool = liver + spleen + ovary pool.

[3]15 d postinoculation = samples were collected from ceca, ovary, and liver + spleen. All the samples from the animals of each group were analyzed individually.

[4]30 d postinoculation = the tissues were analyzed in 2 pools by group: liver + spleen + ovary pool and a ceca pool. In group 4, ceca were analyzed individually.

Eggs. The eggs laid were collected daily. They were analyzed collectively for each group 2 wk before the inoculation, 1 d before the inoculation, and weekly after the challenge.

Feed. Both feeds (with and without the commercial product) were cultured once before the trial started. Twenty-five grams per group was analyzed.

Tissues. Birds were killed at 15 and 29 d postinfection. Postmortem examination tissues were tested in accordance with the Standard Procedure of the Department of Bacteriology of the Centre de Sanitat Avícola de Catalunya i Aragó. At 15 d postinoculation, 5 hens/group were slaughtered to check for systemic infection. In each hen, samples were collected from ceca, ovary, liver, and spleen. All the samples from the animals of each group were analyzed individually. At 29 d postinoculation, again 5 hens/group were slaughtered. The tissues were analyzed in 2 pools by group: liver + spleen + ovary pool and a ceca pool. But in group 4, ceca were analyzed individually.

Blood. Five birds per flock were bled at 17 and 19 wk of age (before the experimental inoculation) and before killing at 15 and 29 d postinoculation. Sera were analyzed for antibody against *Salmonella* using a commercial ELISA kit.

RESULTS AND DISCUSSION

Tables 2, 3, and 4 show the bacteriologic results of isolation and identification of *Salmonella* Enteritidis in each of the groups. Analysis to detect *Salmonella* before the beginning of the trial in compound feed (Table 5) and hens (Table 2, blood) was shown to be negative. In 17-wk-old pullets, the analysis had to be carried out using just 23 individuals due to the bad state of 2 of the blood serum samples. Two positives in group 3 and 1 positive in group 4 were considered to be false positives, because the microbiology results were negative.

Inoculum containing 3.2×10^7 cfu *Salmonella* Enteritidis per hen produced a systemic and intestinal infection with isolation in feces from 1 d postinfection (Table 2), internal tissues and reproductive tracts from 15 d postinfection (Table 2 and 3), and eggs from 4 d postinfection (Table 2). Gast et al. [29, 30] found colonization of the reproductive and internal viscera after an experimental infection with *Salmonella* Enteritidis and *Salmonella* Heidelberg, in which nearly all cecal samples were positive for *Salmonella* at 7 d, as well as 83.3% of the liver samples, 83.3% of spleens, and 66.7% of ovaries. But at 21 d, the percentage of infections in the cecal samples declined by approximately 50%, and in the case of spleens, it ranged from 16.7 to 33.3%, ovaries <16.7%, and no positive results to *Salmonella* were found in liver. Bearing this in mind, viscera checks in the experimental study with the aromatic product were carried out individually 15 d postinoculation to get an overview of *Salmonella* distribution in the hens in each group, but once the systemic infection was proven to exist in every different group, viscera or ceca pools were carried out to determine the evolution of the infection.

Table 4. Serologic results by ELISA[1]

	Group			
Item	1	2	3	4
Serology postinoculation (positivity/samplings)	2/2	0/2	2/2	0/2

[1]Groups: 1 = treated and inoculated; 2 = treated and not inoculated; 3 = not treated and inoculated (positive control); 4 = not treated and not inoculated (negative control).

Experimental oral infection in 19-wk-old pullets produced an intestinal infection with shedding and isolation in feces from d 1 postinoculation and continuously during the whole length of the trial in groups 3 (infected and no treatment) and 1 (infected and treatment; Table 2). The systemic infection was made evident at 15 d postinoculation, with *Salmonella* Enteritidis being detected in liver, spleen, ceca, and serologic tests in groups 3 (infected and no treatment) and 1 (infected and treatment; Table 3). From d 29 postinoculation onward, *Salmonella* was only found in ceca in group 3 (infected and not treated; Table 3).

Differences found among group 1 (infected and treated) and group 3 (infected and not treated) were that in group 1, the results in liver, spleen, and ovary were positive (systemic infection) at 15 d postinoculation but negative from 29 d postinoculation. In group 3, the results were positive for the liver-spleen-ovary pool until 29 d postinoculation (Tables 2 and 3).

The treatment is apparently effective in group 1, because it probably prevents the colonization of ceca and viscera infection. Even though the animals were in a contaminated environment, 29 d postinoculation, the results in liver, spleen, and ovary were negative.

These results are in line with literature data [6, 31, 32] that give rise to the conclusion that clove essential oil and eugenol have an in vitro antimicrobial activity against numerous bacteria, mainly enteric pathogens (*Escherichia coli* and *Salmonella*), with little effect on beneficial gut bacteria. Eugenol showed bactericidal activity against different strains of *Salmonella* [32]. Concerning the antimicrobial mechanisms of essential oils, it has been described that their constituents could get partitioned into the lipid bilayer of the cell membrane, rendering it more permeable, leading to leakage of vital cell contents [33]. The hydroxyl group of eugenol is also thought to bind to membrane proteins [34].

This antimicrobial activity may result in the control of the intestinal colonization by *Salmonella*, which is the point of entrance of the systemic and the reproductive tract infections [35–38]. Moreover, *Salmonella* Enteritidis has selective ability to colonize the reproductive organs, and the different egg parts (yolk, albumen, eggshell membranes, or eggshells) may become directly contaminated by *Salmonella* in the ovary and oviduct [36] before oviposition. The colonization of the ovary or the oviduct in laying hens can be produced by a systemic *Salmonella* Enteritidis infection and also by oral inoculation [35], as it was in the present work. Egg contamination by *Salmonella* Enteritidis can also be caused by penetration through the eggshell from contaminated feces after or during oviposition [35]. In this way, before eggshell deposition, forming eggs are subjected to descending infections from colonized ovarian tissue, ascending infections from colonized vaginal and cloacal tissues, and lateral infections from colonized upper oviduct tissue. The bactericidal action before the eggs are laid (by reduction in the amount of

Table 5. *Salmonella* Enteritidis isolation and identification in environmental samples

	Groups[1]					
Age, wk (days postinoculation)	1	2	3	4	Room sampling	Feed
Before the hens entered, before disinfection	Negative				Negative	Negative
Before the hens entered, after disinfection	Negative				Negative	
21 wk (8 d)	Positive	Negative	Positive	Positive	Positive	
24 wk (30 d)	Positive	Negative	Positive	Negative		

[1]Groups: 1 = treated and inoculated; 2 = treated and not inoculated; 3 = not treated and inoculated (positive control); 4 = not treated and not inoculated (negative control).

pathogen in the reproductive organs) and after deposition (avoiding the contamination with infected feces) can play an important role in the control of *Salmonella* infection.

Differences among groups not infected and treated (group 2) and not infected and not treated (group 4) were observed in the sampling of eggs; group 2 was always negative, whereas group 4 showed positive results at 12 and 19 d postinoculation. In the sampling of feces, group 4 showed positive results on d 1 postinoculation (Table 2). The data compiled demonstrate that the group not treated (group 4) was susceptible to environmental infection, whereas there were no positives in the treated group (group 2), which may be due to a greater resistance to the environmental infection due to the treatment with the aromatic product.

Essential oil of *E. caryophyllata* included in the aromatic product may have prevented cross contamination in eggs. The most important target in the control of a systemic infection is to prevent the presence of *Salmonella* in eggs. In group 1 (inoculated and treated), this was observed, because 19 d postinfection was the last positive result in eggs even though the environment was contaminated. By d 29 postinoculation, the same happened with ceca and viscera, which included ovary (Table 2).

The effect of the aromatic product on intestinal colonization, fecal elimination, systemic infection, and contamination of the eggs seems to be effective in elimination of the intestinal and systemic infection in the group given the essential oil. The effects were detectable 29 d postinoculation. Several internal studies not yet published indicate that the way *E. caryophyllata* works may be by stimulating the cellular immunity (neutrophils, macrophages, CD8 and CD4+ cells), which may encourage inflammatory processes that prevent the colonization of *Salmonella* in the intestine [39–41].

It is also important to point out that in the serologic results in the different groups, especially in group 1, the level of seropositivity observed was nearly 100%, a fact which indicates recent contact with *Salmonella*. Because the culture results were negative, this was considered to be due to postinfection residual immunity (Table 4).

CONCLUSIONS AND APPLICATIONS

1. The aromatic product containing 20% eugenol from clove essential oil (*Eugenia caryophyllata)* added to the feed of the hens prevented cross contamination in eggs.
2. The aromatic product did not prevent the intestinal infection but seems to be effective in wiping out the intestinal and systemic infections.

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