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Antimicrobial and Cytotoxic Bioprospection of *Thymus vulgaris* and Thymol Against *Salmonella enterica* Serovar Heidelberg Isolated in Broiler Chicken

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HIGHLIGHTS

- *Thymus vulgaris* essential oil significantly controlled all studied strains.
- Monoterpene thymol did not control all isolates but showed antibacterial activity.
- *Thymus vulgaris* essential oil showed low cytotoxicity in NCTC fibroblasts.

Abstract: *Salmonella enterica* serovar Heidelberg (SH), often isolated from broiler chicken samples, damages the entire production chain due to its high resistance in the environment. The search for sustainable disinfectant agents has intensified, focusing on the action of essential oils. This work's objective was to evaluate the *in vitro*, effect of *Thymus vulgaris* essential oil (TEO) and thymol in SH isolated from broilers, and the cytotoxicity in animal cells. Four different concentrations of TEO (0.1%, 0.2%, 0.4%, and 0.8%, (v/v)) were used against five SH isolates obtained from broilers and ATCC 8326. Thymol was evaluated at the concentrations of 0.023%, 0.047%, 0.071%, and 0.094% (v/v). *In vitro* antibacterial assays were performed by quantifying viable planktonic cells in the broth microdilution test. The MTT technique was used to assess the cytotoxicity in IEC-6 intestinal cells and NCTC fibroblasts. IC50 was assessed testing the concentrations of 0.0625%, 0.125%, 0.25%, and 0.5% of TEO and thymol in 24 hours. The bacterial activity was observed from the 0.2% concentration of TEO since no colony-forming units were observed. Thymol in the concentration of 0.094% controlled 83.33% of the bacteria. TEO presented an IC50 of 0.14% and 1.22%, while the IC50 for thymol was 0.068% and 0.001% for the IEC-6 and NCTC cells, respectively. On the other hand, TEO showed low cytotoxicity in fibroblasts and a potential action capacity to eliminate SH strains *in vitro*.

Keywords: antibacterial activity; thyme; salmonellosis; sanitizing.

INTRODUCTION

Farm animals, such as chickens and pigs, are predominant host animals for many *Salmonella enterica* serovars. The current intensive rearing system favors the spread and prevalence of this bacterium, which can be considered the focus of enteric salmonellosis epidemiology [1]. Although many infections by non-typhoid salmonella serovars are easily controlled, contamination by *Salmonella enterica* serovar Heidelberg (SH) can cause more evasive infections, such as myocarditis and bacteremia, requiring treatment with third-generation antibiotics [2]. Recent studies have shown that SH is among the most commonly found strains in animal production and products in its chain [3].

The epidemiological complexity and the absence of clinical signs and lesions in chickens infected by paratyphoid serovars mostly determine asymptomatic carriers that can contribute to the product's contamination. This interferes with food security and is concerning factors for the industry and public health. In parallel, it is also worth mentioning bacterial resistance since many studies have recently evaluated the potential of bacterial resistance to antibiotics [4-5] and disinfectants [6-7]. For example, quaternary ammonia is one of the primary disinfecting agents currently used to clean poultry houses [8]. However, *Salmonella* is reported to resist this product [6], which challenges producers in the face of restricted markets regarding the use of antibiotics, making it difficult for the poultry industry to control this serovar.

The safe and sustainable production of the meat, free of *Salmonella*, opens the way to new markets. Several studies in the literature report the antibacterial activity of essential oils (EOs) and their isolated compounds [9-12]. These are substances derived from secondary plant metabolism, such as defense mechanisms of plants against pests and pathogens [13,14].

Thymus vulgaris belongs to the *Lamiaceae* family, native to southern Europe, and popularly known as thyme. Its use as a condiment is disseminated globally [15]. Recent studies have shown promising results in using *T. vulgaris* essential oil (TEO) as antibacterials in *in vitro* tests, especially against gram-negative bacteria [16-18]. Thymol (2-isopropyl-5-methyl-phenol) stands out among the components of this oil and represents more than 50% of the terpenes [16], being commonly the major component of the EO of plants of the *Lamiaceae* family. Thymol is a monoterpene biosynthesized by aromatizing γ-terpinene to p-cymene, followed by the hydroxylation of the p-cymene [19].

The activity of TEO and thymol against SH strains isolated from the field has not been described in the literature focusing on its use as a food additive or disinfectant. Thus, this study's objective was to assess the *in vitro* antibacterial activity of TEO and thymol against SH isolated from broiler chickens and the cytotoxicity in animal cells.

MATERIAL AND METHODS

Essential oil (EO) and thymol

The *Thymus vulgaris* essential oil (TEO) was acquired from the company Ferquima (Vargem Grande, SP, Brazil). The chemical composition, as informed by the supplier, is described in Table 1. Thymol PA was also acquired commercially (Dinâmica, Brazil).

Table 1. Chemical composition of the TEO

Spike	Component	%
1	α-pinene	2.2
2	camphene	0.8
3	myrcene	1.4
4	p-cymene	26.8
5	1,8-cineol	1.3
6	γ-terpinene	6
7	linalool	5.2
8	Canfor	1.5
9	borneol	0.9
10	thymol	47.3
11	carvacrol	3.1
12	β-caryophyillene	0.8

Source: Ferquima

TEO and thymol-based solutions

The solutions were prepared according to the methodology adapted from Millezi [20]. The dilutions were made using a solution containing 2.5% TEO and thymol emulsified in ethanol (solutions made separately, composed of thymol or 2.5% TEO, 2.0% ethanol, and 0.85% saline water (v/v)). TEO was diluted at the concentrations of 0.1%, 0.2%, 0.4%, and 0.8% (v/v) in TSB (Tryptone Soy Broth) medium (Fluka, India) and thymol was diluted at the concentrations of 0.023%, 0.047%, 0.047%, 0.071%, and 0.094% (v/v). The concentrations were based on the amount of thymol present in TEO (47.3%), considering 0.094% of thymol equivalent to the concentration of 0.2% of TEO.

Obtaining, maintaining, and reactivating the bacterial strains

A company of the poultry slaughter and processing sector in the region of the Seara and Ipumirim municipalities (SC, Brazil) provided five strains of SH (already isolated and identified at the originating institution between the years 2017 and 2018). The bacteria were isolated from samples of pre-slaughter drag-swab, collected by drag swabs from broiler beds - the SH strain ATCC 8326. SH strains were kept frozen in a freezer at -80 ºC (preserved in BHI broth end 50% glycerol). To reactivate the bacteria, 10 µL of each culture was inoculated in tubes containing 4 mL of TSB broth (Fluka, India) and incubated for 24 h at 37 °C. After incubation, the inoculum was sown by a simple streak in TSA solid culture medium (Trypticase Soy Agar (Acumedia, Brazil).

Assessment of the antimicrobial activity through CFU quantification

An amount of 10^7 CFU/ml (standardization through a bacterial calibration curv

e) were inoculated into 96-pit microplates, after which the microplates were incubated in BOD (Biochemical Oxygen Demand) incubator (Eletrolab, Brazil) for 24 hours. The treatments of each essay consisted of TEO, thymol, positive control solutions which was composed by bacterial suspension in BHI (Brain Heart Infusion) medium (Oxoid, England) without oil and thymol, and controls containing sterile distilled water to replace the corresponding aliquots of TEO and thymol of each concentration [20].

The planktonic cells were quantified using 100 µL of the supernatant from each pit, serially diluted, and plated in TSA medium using the microtip technique [21]. The plates were incubated at 37 °C. After 24h, plate counting was performed, with values expressed in CFU/mL. Celular cultivation.

For the cytotoxicity tests, fibroblasts of the NCTC line and intestinal epithelial cells of the IEC-6 line (Intestinal Epithelial Cell) were cultured in passages 46 and 23, respectively. The cells were grown in monolayers, in sterile cell culture flasks, maintained in DMEM High medium (Dulbecco's Modified Eagle's Medium, INLAB Diagnóstica), supplemented with 10% FBS (fetal bovine serum, Gibco), and kept in a humid oven at 37 °C with 5% carbon dioxide (CO_2). When cell growth showed an approximate 90% confluence, the cells were removed from the culture flask by trypsinization. This suspension was then centrifuged at 300 g for 5 min, and the pellet containing the cells was resuspended in 1 ml of DMEM High medium. An aliquot was removed from the obtained suspension and quantified in an automatic cell counter (Moxi). Based on the number of cells counted, dilution was performed with DMEM High medium supplemented with 10% FBS to obtain a suspension containing 1×10^5 viable cells/pit. The suspension was dispensed in a 96-pit plate, which was kept for approximately 24 hours in the oven at 37 ºC and 5% CO_2 until the cells' confluence reached approximately 80-90%. Some pits received only 100 µL of RPMI medium (without cells) to serve as a blank and control.

TEO and thymol were emulsified in 0.67% DMSO and 25% ethyl alcohol in the concentration range of 0.5, 0.25, 0.125, and 0.0625%. After the cells acquired confluence, the DMEM medium was discarded, and 100 µL of RPMI 1640 medium was placed in the orange pits (blank assays), 100 µL of RPMI 1640 medium in the pink pits (control wells containing cells), 100 µL of each concentration of the emulsion formulation in the green pits (without cells) and purple pits (containing cells). The plates were placed in a greenhouse with 5% CO_2 at 37 ºC for 24 h.

Assessment of the cytotoxicity using the MTT method

The cells exposed to the emulsions for 24 h were subjected to cell viability assessment by the 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide method (MTT) (Sigma-Aldrich) to verify the 50% inhibitory concentration of cell growth (IC_{50}). Thus, viable cells reduce MTT (yellow) and form formazan (blue colored crystals) [26]. First, the culture medium was removed, and the pits were washed twice with sterile DPBS (Dulbecco′s Phosphate Buffered Saline) at 37 ºC. Subsequently, 90 µl of RPMI and 10 µL of the MTT solution (5 mg/mL) were added to all pits. The plate was incubated for two hours in a humid oven at 37 °C

with 5% CO_2. The medium was then removed, and the plates were subjected to agitation with 100 µL of dimethyl sulfoxide (DMSO, MP Biomedicals) for 5 min. The reading was performed on a spectrophotometer at a wavelength of 540 nm.

Finally, cytotoxicity was calculated according to the equation: [(*a*-*b*)/*c*]x100, where *a* is the absorbance of the sample, *b* is the absorbance of the blank, and *c* is the absorbance of the control. The IC_{50} was calculated based on the equation's results, which is the concentration of the extract that induces 50% of cell lysis through the Prism 8 program (Graph Phad, San Diego, CA).

Statistical analysis

The antibacterial activity results were analyzed using Analysis of Variance (ANOVA) and Dunnet's test to compare the control with the treatment concentrations, considering $P<0.05$ as significant. The analyses were performed in three replicates. For the cytotoxicity test, the treatments were conducted in six replicates, and the averages were analyzed through analysis of variance (ANOVA) followed by the Tukey test ($P<0.05$). All data were analyzed using the Prism 8 software (Graph Phad, San Diego, CA).

RESULTS

TEO significantly controlled all studied strains

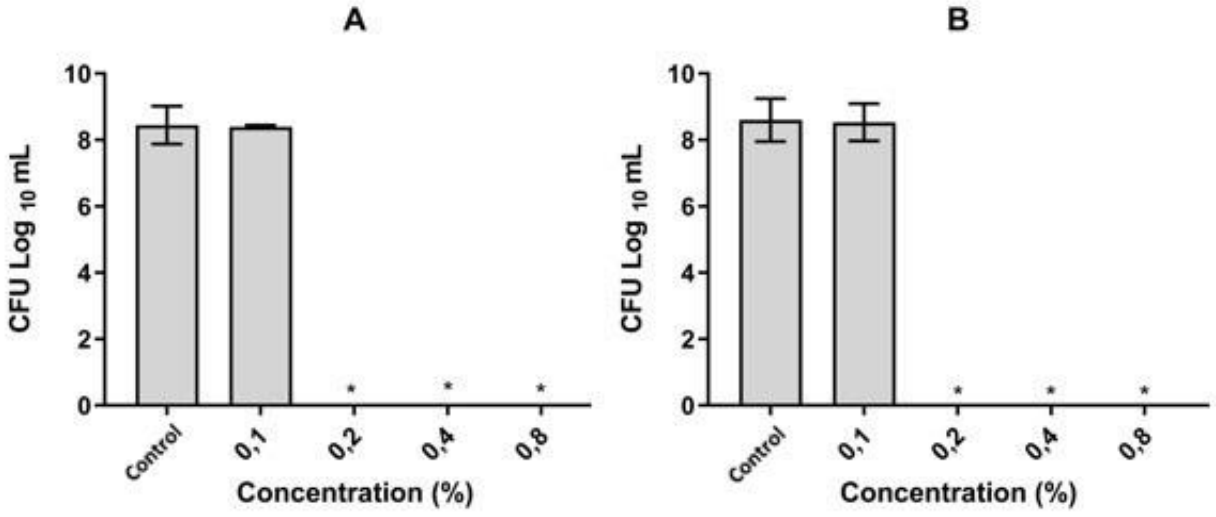
The antibacterial effect of TEO showed significant control of SH from the concentration of 0.2% ($P<0.05$), varying according to the isolate studied. From the concentration of 0.2% TEO, 50% of the strains evaluated had their growth reduced. The other 50% of the bacteria showed a growth reduction from 0.1% of TEO (Figure 1).

Monoterpene thymol did not control all isolates

Thymol showed a significant difference in the growth control of SH isolates. Two of the six strains presented reduced growth from the concentration of 0.047% (E and F), one strain from the concentration of 0.071% (A), two strains from the concentration of 0.094% (C and D), and one strain was not inhibited at the tested concentrations (B). However, it is worth noting that 83.33% of the strains tested showed inhibition of bacterial growth at the concentration of 0.094% thymol (Figure 2).

TEO showed low cytotoxicity in NCTC fibroblasts

The cell viability test in IEC-6 cells showed some toxicity in the tested concentrations. The IC_{50} of TEO was 0.14% in 24 h and 0.069%for thymol (Figure 3). On the other hand, when the same substrates were exposed to NCTC fibroblasts, the IC_{50} of TEO was 1.23% and of thymol was 0.001% (Figure 4), showing less toxicity of TEO in this cell type but high cytotoxicity to thymol.



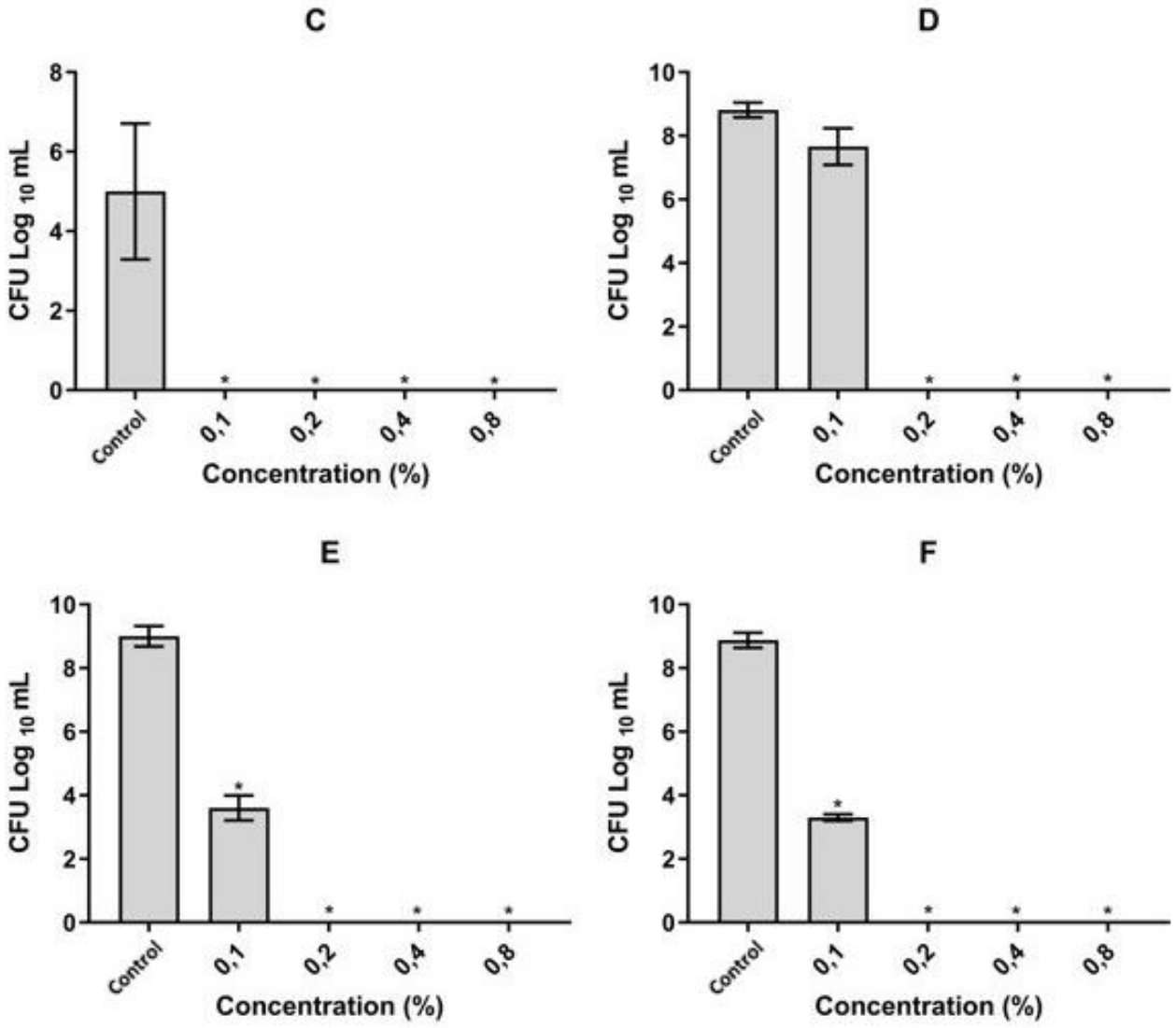
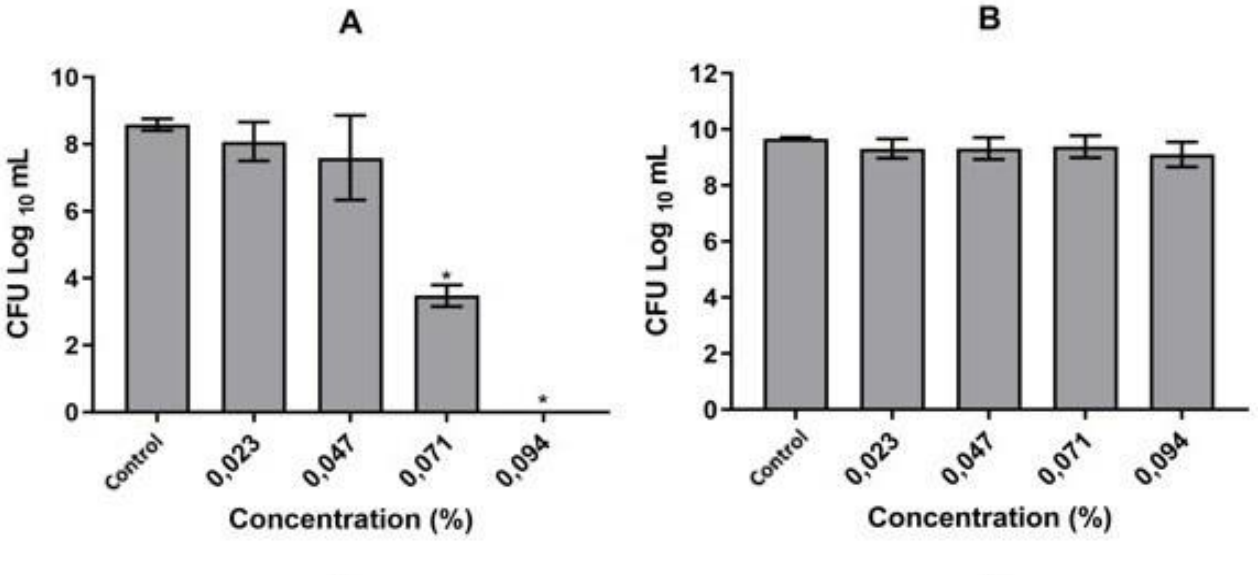


Figure 1. Growth of SH CFUs under treatments of different concentrations of TEO. (A) SH ATCC 8326; (B) SH SI01; (C) SH SI02; (D) SH SI03; (E) SH SS01; (F) SH SS02.*P<0.05 by Dunnett's test (ANOVA).



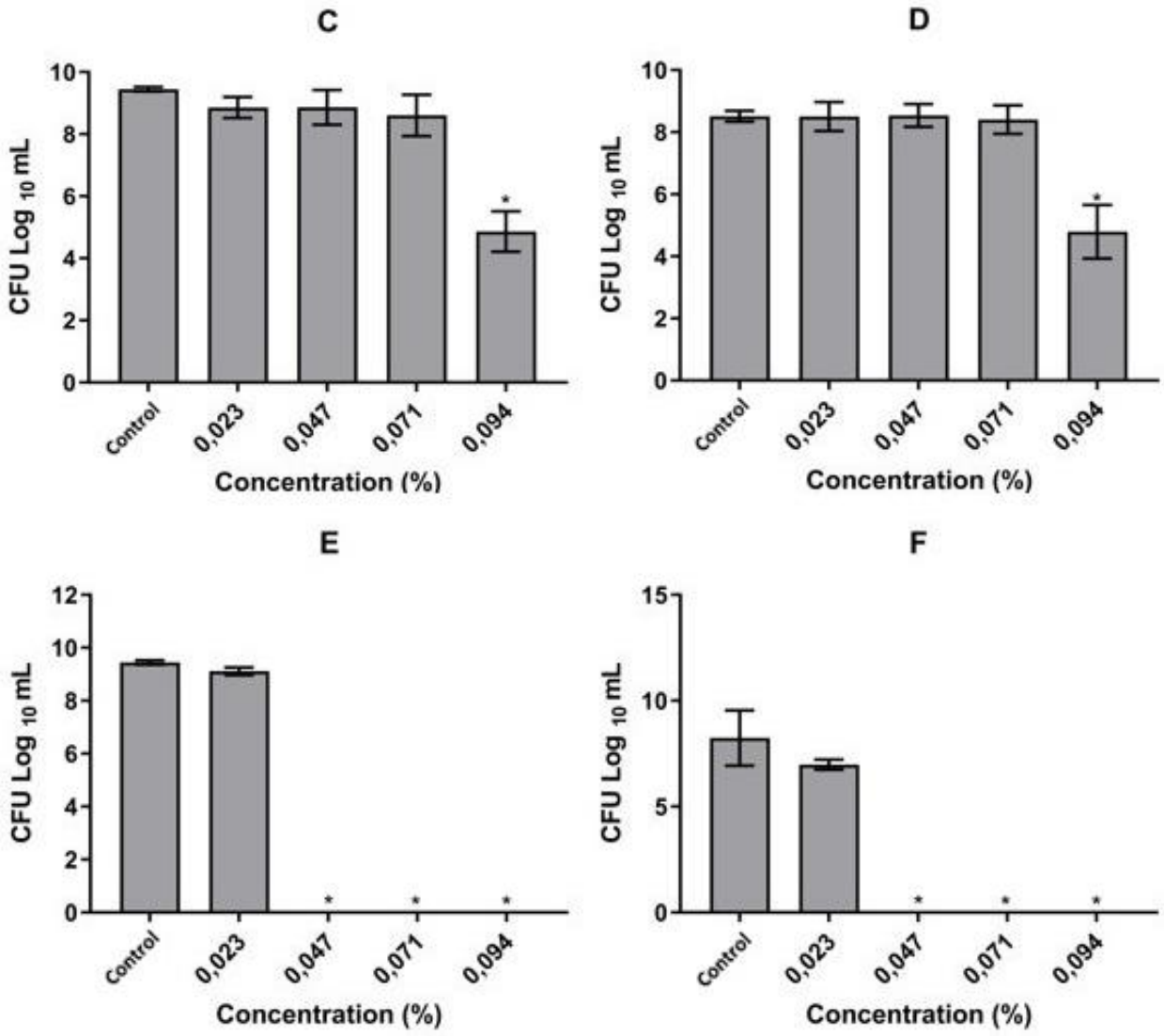


Figure 2. Growth of SH CFUs under treatments of different concentrations of thymol. (A) SH ATCC 8326; (B) SH SI01; (C) SH SI02; (D) SH SI03; (E) SH SS01; (F) SH SS02.*P<0.05 by Dunnett's test (ANOVA).

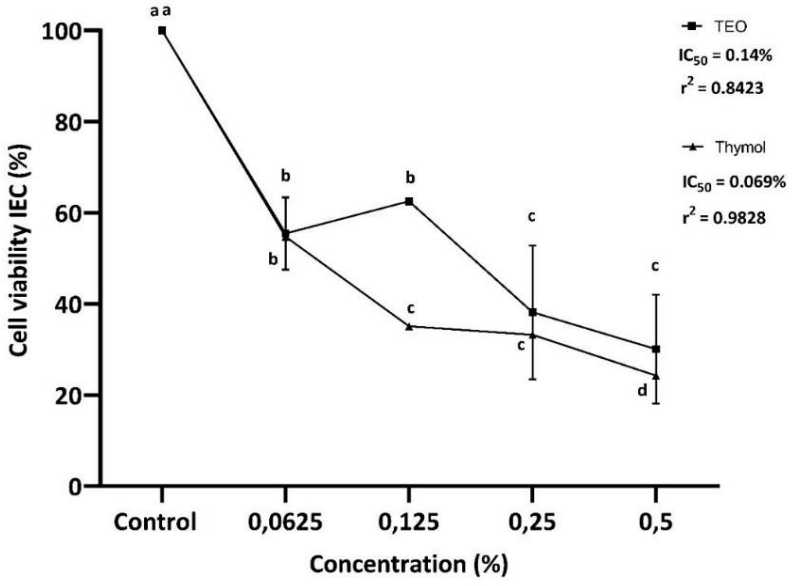


Figure 3. Viability of IEC-6 cells after the exposure to TEO and thymol at the concentrations of 0.5%, 0.25%, 0.125%, and 0.0625% in 24 h. Different lowercase letters represent statistical differences between the concentrations (P<0.05).

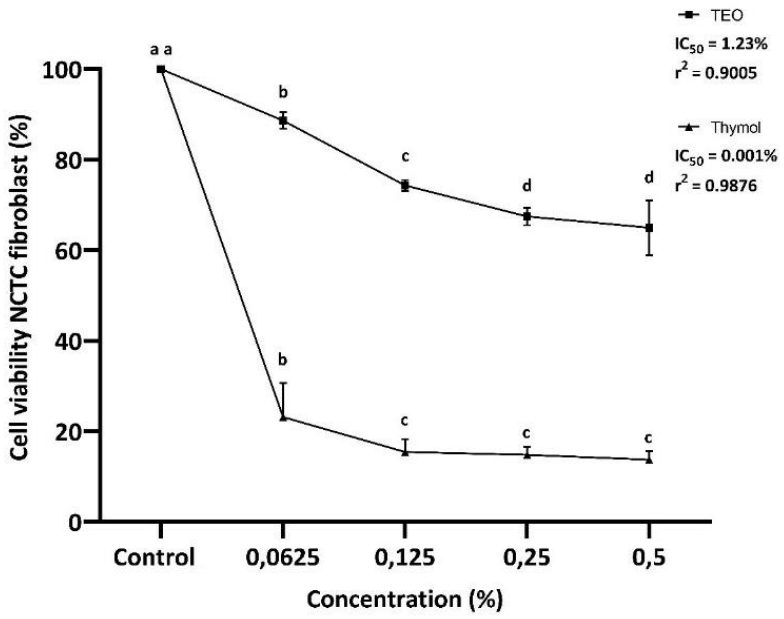


Figure 4. Viability of Fibroblast-NCTC cells to TEO and thymol at the concentrations of 0.5%, 0.25%, 0.125%, and 0.0625% in 24 h. Different lowercase letters represent statistical differences between the concentrations (P<0.05).

DISCUSSION

In recent years, SH has been among the *Salmonella* strains most prevalent in animal production and its chain products [3]. This bacterium's resistance to antibiotics and the formation of bacterial biofilms are concerning to the industry and public health [22]. Recently, many studies have demonstrated the resistance of this bacterium to antibiotics. Gieraltowski [5] evaluated the resistance of 69 SH samples collected from sick patients and found that 67% of the strains were resistant to antibiotics, and of those, 35% were multidrug-resistant. Taylor [4] isolated nine SH samples and found that two were multidrug-resistant to cephalosporin and ceftriaxone, which are third-generation antibiotics. Bacterial resistance challenges producers to improve their production in the face of market restrictions on antimicrobials in poultry, mainly as growth promoters, making it difficult for the poultry industry to control many microorganisms, including *Salmonella*.

There are many studies on the antibacterial activity of EOs [9-12], especially with TEO [16-18] and thymol [23,24]. However, research on the activity of the compounds used in this work against SH is scarce. Peichel [28] tested the effect of the *Cymbopogon citratus* (lemongrass) essential oil against strains of multi-resistant SH, inoculated in the drinking water of broilers, and found the inhibition of bacterial growth at the concentration of 0.25% of the EO in 24h. In this study, TEO at a concentration of 0.2% could control the bacterial growth of 100% of the tested SH strains. Boskovic [16] evaluated the effect of TEO against *S. enteritidis* and *S. thyphimurium*, reporting an inhibition at the concentration of 0.032%, lower than the concentrations used in this study. The low activity is probably due to the difference in the strains tested or the slightly different composition of the oil used (composed of 50.48% thymol; 24.79% p-cymene; 4.69% linalool; 4.14 % of γ-terpinene; and 4.35% of 1.8-cineole). However, this proves the possibility of using TEO against other species of *Salmonella*.

The TEO tested in this study controlled all strains, with 47.3% of thymol, proportionally, in the concentration of 0.2% of TEO. On the other hand, Thymol showed an antibacterial effect in 83.33% of the strains studied at a concentration of 0.094%. This result highlights that thymol alone was not as efficient as TEO in the face of the studied SH strains. There is evidence of synergism between the monoterpenes present in the essential oils [12,25]. This is probably why the TEO showed an antimicrobial activity significantly better than thymol. Donato [25] found that the *Artemisia annua* essential oil and three of the major components of the oil (isoartemisiacetone, eucalyptol, and camphor) decreased the bacterial growth of *Salmonella enteritidis* (ATCC 13311) and *Salmonella typhi* (ATCC 19430) using lower concentrations when compared to its major component, terpene.

The variation of the inhibitory effect found between the strains may occur due to genetic variation since five field isolates from different poultry farms, and the ATCC strain were evaluated. Genetic variations between bacteria from the same family are quite common since strains isolated in the field tend to have greater virulence and resistance because they are subject to environmental diversity. Sperandio [12] evaluated the *Tagetes minuta* essential oil against strains of *Escherichia coli* isolated from mastitic milk and

found a statistical difference in the minimum inhibitory concentration between the ATCC strain and the isolates. The *E. coli* standard strain, on the other hand, showed inhibition in 1 mg/mL of the tested oil and growth reduction of the isolated bacteria only at concentrations superior to 3 mg/mL.

The challenge in using essential oils and their compounds is adjusting the concentrations that are effectively antibacterial and not cytotoxic to animals and humans. The concentration that inhibits 50% of cell growth (IC_{50}) showed higher cytotoxicity of thymol to the cell types used in this work compared to TEO. The IC_{50} for thymol was 0.069% and 0.001% in IEC-6 cells and fibroblasts, respectively. It is unfeasible to use this substrate in broilers, both internal and external, when comparing these results with the concentration of bacterial growth inhibition of thymol, which was 0.094%. Some studies demonstrate that thymol increases the incidence of cell death due to apoptosis when tested in Caco-2 cells (human intestinal cells) at the same concentrations in which they have a good antimicrobial effect [23,24].

The IC_{50} for TEO was 0.14% for IEC-6 cells and 1.23% for fibroblasts, which were superior to the values found for thymol. However, considering that the concentration of 0.2% TEO showed 100% inhibition of SH bacterial growth, the use of this essential oil is not recommended for internal treatment to reduce SH growth. In contrast, a low cytotoxicity of the essential oil was found in fibroblast cells. Oliveira [27] analyzed different concentrations of *Thymus vulgaris* essential oil extracts from in human gingival fibroblasts (FMM-1), macrophages (RAW264.7), breast carcinoma cells (MCF-7), and uterine carcinoma cells (HeLa). After 5 min of exposure to the extract, the authors found that the highest concentration tested (1.0%) showed cell viability above 50%. The present study showed that a concentration of 1.23% of essential oil is necessary to inhibit 50% of cell growth, a concentration six times higher than the minimum necessary to inhibit all strains of SH tested. These studies show that SH is a strain susceptible to essential oils and that these compounds can reduce/inhibit SH's growth in broiler chickens.

CONCLUSION

The TEO controlled all *Salmonella enterica* serovar Heidelberg bacteria at the concentration of 0.2%, showing low cytotoxicity to fibroblasts. On the other hand, the monoterpene thymol did not control all the strains studied and indicated *in vitro* cytotoxicity concerning the cell lines evaluated. Studies evaluating intermediate concentrations should be conducted to better understand the effect of TEO and thymol against this bacterial type.

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