



Emulsions of essential oils in challenged meat with *Salmonella enterica* subsp. *enterica* serotype Enteritidis

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[Emulsões de óleos essenciais em carne desafiada com *Salmonella enterica* subsp. *enterica* sorovar Enteritidis]

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ABSTRACT

The objective of this project was to evaluate the antimicrobial activity of essential oil (EO) emulsions in different types of meat challenged with *Salmonella enterica* subsp. *enterica* serotype Enteritidis (SE). Concentrations of 0.0%, 1.0%, 1.5%, and 2.0% of EO emulsions from eucalyptus (*Eucalyptus globulus*), lemon (*Citrus limon*), clove (*Syzygium aromaticum*), and oregano (*Origanum vulgare*) were prepared in chicken meat, beef, and pork inoculated with SE at 1-hour and 24 hours. The effectiveness of EO against SE in different types of meat showed the greatest bacterial reduction in beef and with the exposure time of 24 hours. The effectiveness of eucalyptus EO against bacterial growth was shown to be inferior compared to oregano, clove, and lemon EO. Therefore, the terpenoid carvacrol, the phenylpropene eugenol, and the terpene limonene demonstrated a greater reduction in SE survival than the 1,8-cineole in eucalyptus. In the interactions, oregano EO showed no differences in chicken meat but did show differences in beef at 1.0% and 1-hour exposure, compared to any concentration in chicken or pork at 1-hour and 24 hours exposure. Eucalyptus EO at 2.0%, 1-hour exposure, and in beef showed differences compared to any concentration in chicken for both exposure times. Increasing the concentration of lemon EO in chicken meat appeared to reduce its effectiveness against SE. Finally, clove EO showed its best results in beef at 1.5% and after 24 hours of exposure. It is recommended to conduct an experiment testing mixed oregano and clove EOs to enhance their bactericidal effect.

Keywords: essential oils, *Salmonella*, meat, eucalyptus, lemon, cloves and oregano.

RESUMO

O objetivo deste projeto foi avaliar a atividade antimicrobiana de emulsões de óleo essencial (OE) em diferentes tipos de carne desafiadas com *Salmonella enterica* subsp. *enterica* sorovar Enteritidis (SE). Concentrações de 0.0%, 1.0%, 1.5% e 2.0% de emulsão de OE de eucalipto (*Eucalyptus globulus*), limão (*Citrus limon*), cravo (*Syzygium aromaticum*) e orégano (*Origanum vulgare*) foram preparadas em carne de frango, carne bovina e carne suína inoculadas com SE em 1h e 24h. A eficácia do OE sobre o SE em diferentes tipos de carne, registrou a maior redução bacteriana na carne bovina e com tempo de exposição de 24h. A eficácia do OE de eucalipto sobre o crescimento da bactéria mostrou-se menor em comparação ao OE de orégano, cravo e limão. Portanto, o terpenoide carvacrol, o fenilpropeno eugenol e o terpeno limoneno apresentam maior redução na sobrevivência do SE do que o 1,8-cineol do eucalipto. Nas interações, o OE de orégano não apresentou diferenças na carne de frango, mas apresentou na carne bovina a 1.0% e 1h de exposição, quando comparado com qualquer concentração na carne de frango ou carne suína por 1h e 24h de exposição. OE de eucalipto a 2.0%, 1h de exposição e na carne bovina, apresentou diferenças quando comparado com qualquer concentração na carne de frango para ambos os tempos de exposição. O aumento da concentração do OE de limão na carne de frango parece reduzir sua eficácia no SE. Finalmente, OE de cravo apresentou seus melhores resultados na carne bovina, a 1.5% e por 24h de exposição. Recomenda-se realizar um experimento onde OEs mistos de orégano e cravo sejam testados, para potencializar seu efeito bactericida.

Palavras-chave: óleos essenciais, *Salmonella*, carne, eucalipto, limão, cravo e orégano

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INTRODUCTION

Synthetic antimicrobials used in food preservation present a high toxicological level (Ju *et al.*, 2022). Therefore, they may be unsafe for human consumption (Reyes *et al.*, 2020). Essential Oils (EOs) are secondary metabolites extracted from plants (Falleh *et al.*, 2020; Sharma *et al.*, 2021). Thanks to their low toxicological level, pharmacological activity, and economic viability, their acceptance as natural antimicrobials by consumers is increasing (Angane *et al.*, 2022). Gavahian *et al.* (2020) noted that EOs are small intercellular droplets formed in the cytoplasm of plant eukaryotic cells. They consist of compounds such as esters, alcohols, aromatic hydrocarbons, terpenes, terpenoids, ketones, acids, and aldehydes (Santos *et al.*, 2022). EOs are primarily extracted from: i) eucalyptus (*Eucalyptus globulus*) (Mieres *et al.*, 2021), ii) clove (*Syzygium aromaticum*) (Haro *et al.*, 2021), iii) lemon (*Citrus limon*) (Singh *et al.*, 2021), iv) oregano (*Origanum vulgare*) (Bora *et al.*, 2022). Although the food industry uses EOs as flavoring agents, their potential as natural antimicrobials has not been fully explored. Among foodborne diseases (FBDs), the Gram-negative bacterium *Salmonella enterica* subsp. *enterica* serotype Enteritidis (SE) is the main facultative intracellular etiological agent worldwide (Ruvalcaba *et al.*, 2022). Synthetic antimicrobials have been the primary strategy for controlling SE (Morasi *et al.*, 2022). However, due to bacterial adaptation and resistance, the use of natural alternatives has been recommended (Falleh *et al.*, 2020). Therefore, the objective of the present research is to evaluate the antimicrobial activity of EOs in different types of meat challenged with SE.

MATERIALS AND METHODS

All procedures used were approved by the Bioethics and Animal Welfare Committee of the Faculty of Veterinary Medicine and Animal Husbandry at the University of Colima (Evaluation Record: No. 004/2023). The study was conducted in the Poultry Laboratory within the postgraduate area of the Faculty of Veterinary Medicine and Animal Husbandry at the University of Colima, located in Tecomán, Colima, at geographic coordinates 18°56'50.3" N, 103°53'45.0" W, at an altitude of 48 m s. n. m. The average temperature is 26°C, with a

precipitation range of 600-1,100 mm. The climate is warm sub-humid with summer rains (Köppen classification: Cfb) (García, 1973; Peel *et al.*, 2007).

A strain of SE obtained from Arkansas State University was used. Its incubation and purification were carried out following the technique described by Tellez *et al.* (1993): i) Incubation of 300 µL of SE in 30 mL of nutrient broth (210300; BD Bioxon, Madrid, Spain) at 37°C for 24 hours; ii) Centrifugation at 3,500 RPM for 4 min using a portable centrifuge (HKSC-220; Globe Scientific, New Jersey, USA); iii) Reconstitution of the sediment with Physiological Saline Solution (PSS). To determine the SE concentration, the McFarland turbidity scale (KMT555; Microkit Laboratories, Madrid, Spain) was used, corresponding to a density of 1.5×10^5 CFU/mL.

The raw materials were sourced from the local market in Tecomán, Colima. Lemon, clove, eucalyptus, and oregano EOs were extracted via steam distillation. An 8L pressure cooker with a grate, 2L of distilled water, and 800g of raw material were used. To generate steam and release the EO, the mixture was heated to 150°C. The steam was directed toward the condenser to collect the EO. The total distillation time was 1-hour and 30 min, yielding 1 mL of EO (see Tables 1 and 2).

The emulsions were prepared using Tween80^{MR} (TW0330; LABESSA, Mexico City, Mexico) with an HLB (Hydrophilic-Lipophilic Balance) of 15.0 and Span80^{MR} (8.40123.0100, Sarsfield Rd., Co. Cork, Ireland) with an HLB of 4.30. Based on the HLB, emulsions were prepared at concentrations of 1.0%, 1.5%, and 2.0% for each EO. A T18 digital ultra-turrax homogenizer (0003720001; Wilmington, USA) was used at 10,000 RPM for 5 min. A total of 50mL of water/oil emulsions were made for lemon, eucalyptus, clove, and oregano EOs at concentrations of 1.0%, 1.5%, and 2.0% (see Table 3).

A total of 60 cuts, each measuring 1cm³, of chicken, beef, and pork were prepared, sourced from the local market (four cuts per type of meat with five replicates each). The experimental groups (Table 4) were assigned to control (0.0%) and treatments with emulsions of lemon,

eucalyptus, clove, and oregano essential oils at concentrations of 1.0%, 1.5%, and 2.0%.

Four sterile Petri dishes were used for each type of meat (chicken, beef, and pork). Each dish was labeled for the concentrations of 0.0%, 1.0%, 1.5%, and 2.0%. For the 0.0% concentration, 5mL of PSS was added, while for the 1.0%, 1.5%, and 2.0% concentrations, 5mL of each

essential oil emulsion (lemon, eucalyptus, clove, and oregano) was added. Additionally, four Petri dishes were labeled for SE and filled with 5mL (10^5 CFU/mL) of SE. The meat cuts (chicken, beef, and pork) were immersed in the 0.0%, 1.0%, 1.5%, and 2.0% EO concentrations. After drying, they were treated by immersion in the dishes with SE for 30sec, followed by drying for an additional 30sec.

Table 1. Chemical composition of essential oils (A)

Eucalyptus (<i>Eucalyptus globulus</i>)		Oregano (<i>Origanum vulgare</i>)	
Compound	Percentage (%)	Compound	Percentage (%)
α -thujene	0.40	α -thujene	2.52
α -pinene	7.30	α -pinene	3.80
camphene	0.80	camphene	3.30
sabinene	1.00	β -pinene	1.71
β -pinene	3.00	α -phellandrene	1.87
β -myrcene	1.70	p-cymene	15.30
α -phellandrene	1.00	γ -terpinene	8.21
α -terpinene	1.00	linalool	9.71
p-cymene	7.70	camphor	1.77
α -limonene	7.00	borneo	1.76
1,8-cineole	63.90	terpinen-4-ol	1.66
(e)-b-ocimene	0.20	thymol methyl ether	1.78
g-terpinene	3.60	carvacrol methyl ether	1.74
α -terpinolene	0.60	thymol	8.60
menthol	0.20	carvacrol	29.60
4-terpinenol	0.20	caryophyllene oxide	6.67
aromadendrene	0.40		

Table 2. Chemical composition of essential oils (B)

Lemon (<i>Citrus limon</i>)		Clove (<i>Syzygium aromaticum</i>)	
Compound	Percentage (%)	Compound	Percentage (%)
limonene	49.54	eugenol	87.30
α -pinene	32.50	eugenyl acetate	10.40
β -citral	4.65	β -caryophyllene	1.35
β -myrcene	4.01	α -humulene	0.19
neryl acetate	1.74	caryophyllene oxide	0.25
β -bisabolene	1.30	chavicol	0.31
α -terpineol	1.26	methyl salicylate	0.08
terpinolene	1.10	benzaldehyde	0.07
α -bergamotene	0.97	benzyl acetate	0.05
tujane	0.85		
caryophyllene	0.72		
4-terpineol	0.68		
geraniol	0.68		

Table 3. Essential oil, Tween80^{MR}, Span80^{MR} and distilled water in the concentrations of 1.0%, 1.5% and 2.0%

Concentration (%)	Essential oil	Tween80MR (g)	Span80MR	Water
Eucalyptus				
1.0	0.50	1.59	0.90	47.0
1.5	0.75	1.59	0.90	46.7
2.0	1.00	1.59	0.90	46.5
Lemon				
1.0	0.50	1.13	1.36	47.0
1.5	0.75	1.13	1.36	46.7
2.0	1.00	1.13	1.36	45.5
Clove				
1.0	0.50	2.25	0.22	47.0
1.5	0.75	2.25	0.22	46.7
2.0	1.00	2.25	0.22	46.5
Oregano				
1.0	0.50	2.27	0.22	47.0
1.5	0.75	2.27	0.22	46.7
2.0	1.00	2.27	0.22	46.5

Tween80^{MR} (TW0330; LABESSA, Mexico City, Mexico); Span80^{MR} (8.40123.0100, Sarsfield Rd., Co. Cork, Ireland).

Table 4. Experimental design to evaluate the biological activity of essential oils on *Salmonella enterica* subsp. *enterica* serotype Enteritidis in different types of meat

	Concentration (%)	B: type of meat		
		Chicken	Beef	Pork
A: essential oils: eucalyptus, lemon, clove, and oregano	0.0	1	21	41
		2	22	42
		3	23	43
		4	24	44
		5	25	45
	1.0	6	26	46
		7	27	47
		8	28	48
		9	29	49
		10	30	50
	1.5	11	31	51
		12	32	52
		13	33	53
		14	34	54
		15	35	55
	2.0	16	36	56
		17	37	57
		18	38	58
		19	39	59
		20	40	60

Different resealable bags, identified by EO type and concentration, were used. The samples (meat cuts) were placed in the bags along with 10mL of PSS and macerated. Subsequently, SE was

quantified using serial dilutions. For this, 200µL of inoculum (macerated meat samples) was added to five wells and 180µL of PSS was added to 15wells in sterile 96-well microplates (6-2013;

Simport Scientific, Saint-Mathieu-de-Beloeil, Canada). Four dilutions were performed for each concentration using a micropipette: 20µL of the inoculum was taken for the second dilution, 20µL for the third dilution, and 20µL for the fourth dilution. Brilliant green agar, supplemented with 20µg/mL of nalidixic acid (N-4382; Sigma-Aldrich, St. Louis, Missouri, USA) and 25µg/mL of novobiocin (N-1628; Sigma-Aldrich, St. Louis, Missouri, USA) (Thung *et al.*, 2016), was used to eliminate contaminating microorganisms. Each sample was plated in triplicate for 1-hour and 24 hours exposure times and incubated in a bacteriological incubator (Bacteriological Incubator 35411; Quincy Lab, Chicago, USA) at 37°C for 24 hours. After incubation, bacterial colony counts were performed.

Colony counts were converted to $\log_{10}$ (N), where N represents the SE population at a given

time (CFU/mL). The data were subjected to a factorial analysis of variance (ANOVA) with a 2 x 3 x 4 design (A x B x C) and five replicates using PROC ANOVA (SAS..., 2001). The factors studied included:

- A. 1-hour and 24 hours exposure times;
- B. Chicken meat, beef, and pork inoculated with SE;
- C. Treatments with EO of lemon, eucalyptus, clove, and oregano at 0.0%, 1.0%, 1.5%, and 2.0%.

A Tukey multiple comparison test was performed to identify differences between groups ($P < 0.05$).

RESULTS

The descriptive statistics for the type of meat, type of EO, exposure time, and EO concentration are shown in Table 5.

Table 5. Effectiveness of essential oils on *Salmonella enterica* subsp. *enterica* serotype *Enteritidis* in different types of meat

	n	Media	DE	EE	CI*		
Type of exposure (h)							
1	720	3.46	1.82	0.06	3.33	–	3.59
24	720	3.10	1.80	0.06	2.97	–	3.24
Type of meat							
Chicken	480	4.02	1.83	0.08	3.88	–	4.17
Beef	480	2.17	1.38	0.06	2.02	–	2.31
Pork	480	3.65	1.65	0.07	3.50	–	3.80
Type of essential oil							
Oregano	360	3.17	1.79	0.09	2.99	–	3.36
Eucalyptus	360	3.54	1.77	0.09	3.35	–	3.73
Lemon	360	3.34	1.81	0.09	3.15	–	3.53
Clove	360	3.07	1.87	0.09	2.88	–	3.26
Oregano oil (%)							
0.0	90	3.64	1.89	0.19	3.27	–	4.01
1.0	90	3.04	1.73	0.18	2.67	–	3.41
1.5	90	3.09	1.71	0.18	2.72	–	3.46
2.0	90	2.92	1.78	0.18	2.55	–	3.27
Eucalyptus oil (%)							
0.0	90	3.91	1.68	0.17	3.54	–	4.28
1.0	90	3.54	1.66	0.17	3.18	–	3.91
1.5	90	3.24	1.80	0.19	2.87	–	3.60
2.0	90	3.48	1.88	0.19	3.11	–	3.85
Lemon oil (%)							
0.0	90	3.08	1.76	0.18	2.70	–	3.45
1.0	90	3.10	1.82	0.19	2.73	–	3.48
1.5	90	3.54	1.87	0.19	3.17	–	3.91
2.0	90	3.63	1.77	0.18	3.26	–	4.01
Clove oil (%)							
0.0	90	3.27	1.60	0.16	2.88	–	3.66
1.0	90	3.09	1.46	0.15	2.70	–	3.48
1.5	90	2.89	2.14	0.22	2.50	–	3.28
2.0	90	3.03	2.17	0.22	2.65	–	3.42

*Confidence interval (95%)

The effectiveness of oregano EO and eucalyptus EO against SE, based on the interaction of different concentrations, two exposure times, and different types of meat, is presented in Figures 1 and 2.

The effectiveness of lemon EO and clove EO against SE, based on the interaction of different concentrations, two exposure times, and different types of meat is presented in Figures 3 and 4.

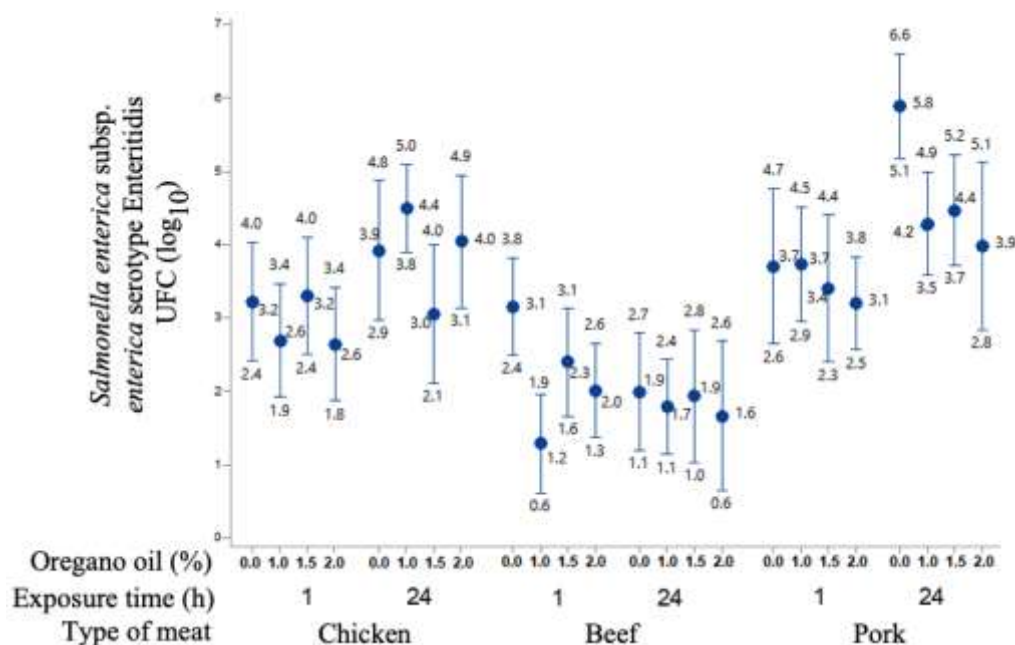


Figure 1. Comparison of the effectiveness of oregano oil on *Salmonella enterica* subsp. *enterica* serotype Enteritidis by interaction of different concentrations, two exposure times, and different types of meat.

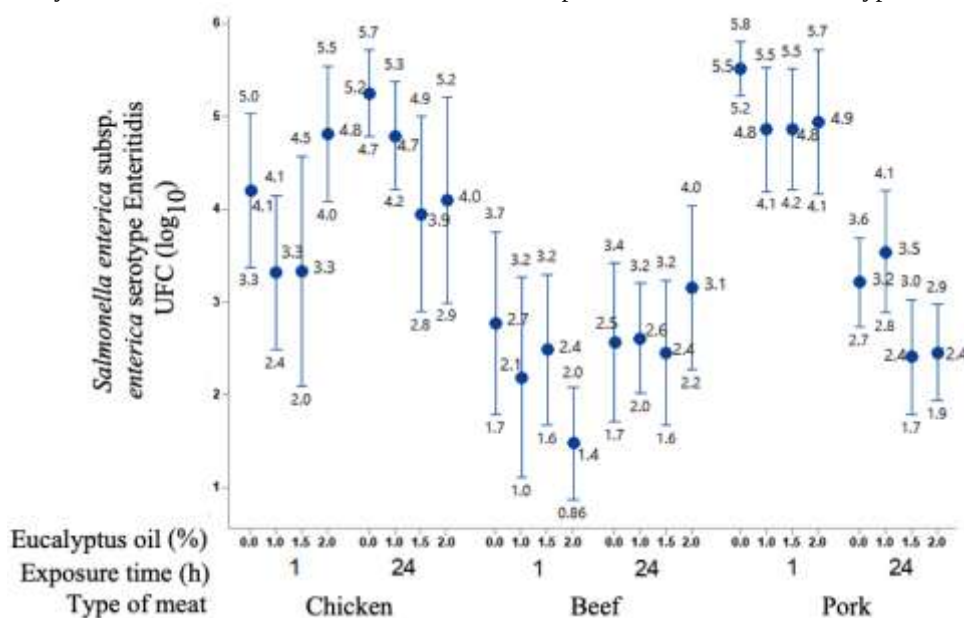


Figure 2. Comparison of the effectiveness of eucalyptus oil on *Salmonella enterica* subsp. *enterica* serotype Enteritidis by interaction of different concentrations, two exposure times, and different types of meat.

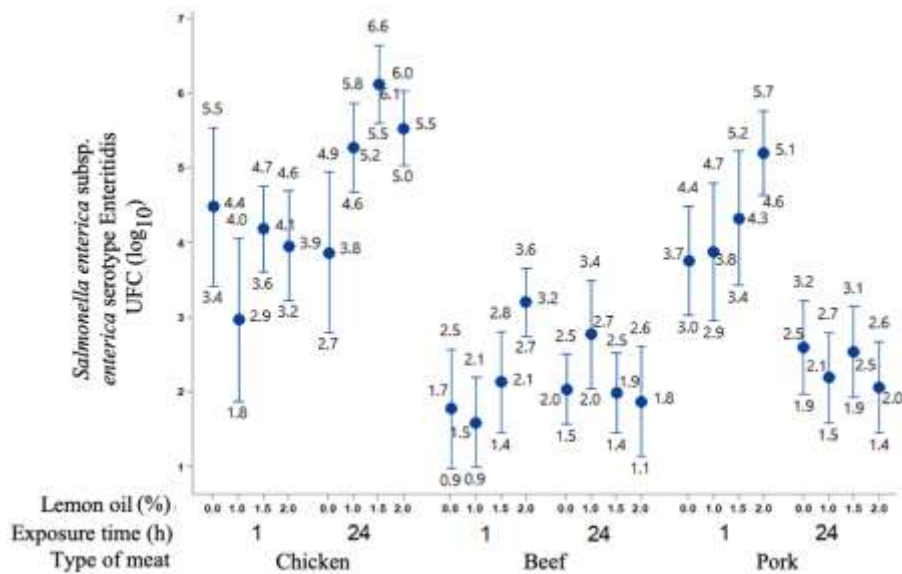


Figure 3. Comparison of the effectiveness of lemon oil on *Salmonella enterica* subsp. *enterica* serotype Enteritidis by interaction of different concentrations, two exposure times, and different types of meat.

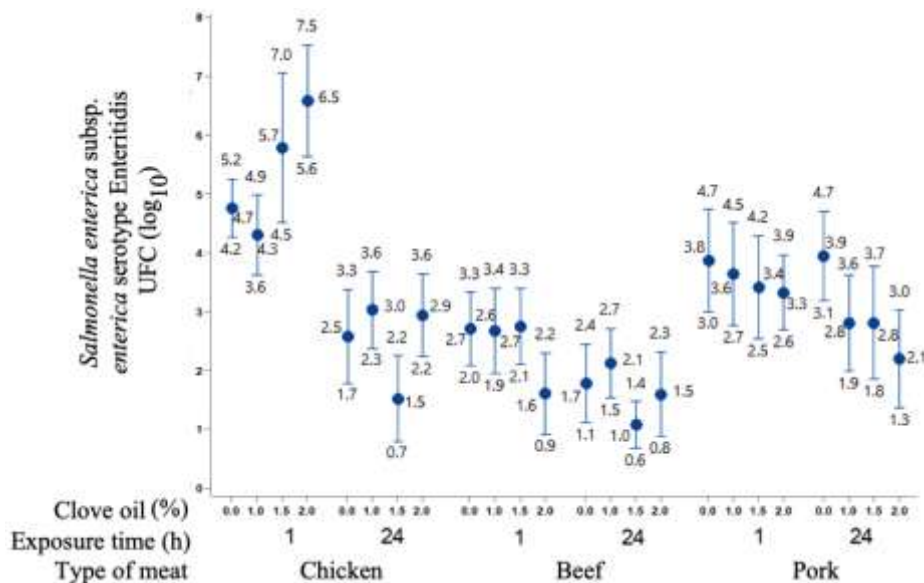


Figure 4. Comparison of the effectiveness of clove oil on *Salmonella enterica* subsp. *enterica* serotype Enteritidis by interaction of different concentrations, two exposure times, and different types of meat.

DISCUSSION

The effectiveness of lemon, eucalyptus, clove, and oregano EOs on SE in different types of meat showed the highest bacterial reduction in beef. This result suggests that beef has a characteristic that, in combination with the studied EOs, affects the survival of SE. Dominguez *et al.* (2019) pointed out that this characteristic is the concentration of the pigment

protein myoglobin, present in muscle myofibrils. Wicks *et al.* (2019) noted that 90% of the iron in meat exists as hemoglobin, myoglobin, and low amounts of ferritin. They also mentioned that myoglobin concentration is higher in beef, giving it a redder color compared to chicken meat and pork (Wicks *et al.*, 2019). The effectiveness of eucalyptus EO on SE was lower ($P < 0.05$) compared to oregano, clove, and lemon EOs. Table 1 shows the main compounds in oregano

EO: i) carvacrol, ii) p-cymene, iii) linalool, and iv) thymol, at concentrations of 29.60%, 15.30%, 9.71%, and 8.60%, respectively. In clove EO, the compounds are: i) eugenol, ii) eugenyl acetate, and iii) β -caryophyllene, at concentrations of 87.30%, 10.40%, and 1.35%, respectively (Table 2). In lemon EO, the main compounds are: i) limonene and ii) α -pinene, at 49.54% and 32.50%, respectively (Table 2). In eucalyptus EO, the compounds are: i) 1,8-cineol, ii) p-cymene, and iii) α -pinene, at 63.90%, 7.70%, and 7.30%, respectively (Table 1). Therefore, the terpenoid carvacrol, the phenylpropene eugenol, and the terpenoid limonene show a greater reduction in the survival of SE compared to the 1,8-cineol in eucalyptus. In this study, the terpenoid carvacrol from oregano EO showed no differences within the group (chicken meat) at any concentration or between the two exposure times. At a concentration of 1%, with 1-hour of exposure, and in beef, this EO showed differences ($P<0.05$) when compared to any concentration in chicken meat or pork for both exposure times. Carvacrol from oregano EO at concentrations of 1.0%, 1.5%, and 2.0% with 24 hours of exposure showed differences ($P<0.05$) when compared to the 0.0% control group. Shange *et al.* (2019) used oregano EO to preserve previously frozen black wildebeest meat, obtaining similar results to ours with a concentration of 1.0% and 9 days of storage at 2°C. Motta Felício *et al.* (2021) developed and characterized a nanoemulsion of carvacrol and evaluated its antimicrobial activity against foodborne pathogens. They found that nanoemulsion with 3% carvacrol EO, 9% surfactants (HLB 11), and 88% water presented enhanced antimicrobial activity compared to free EO.

The 1,8-cineol from eucalyptus EO showed no differences within the group (chicken meat) at any concentration or between the two exposure times. This EO at a concentration of 2.0%, with 1-hour of exposure and in beef, showed differences ($P<0.05$) when compared to any concentration in chicken meat for both exposure times. In comparison to pork, it showed differences ($P<0.05$) at 1-hour of exposure, but only at 1-hour. Guimarães *et al.* (2019) selected antibacterial substances present in different EOs and determined that only 16 out of 33 compounds had antimicrobial activity, with eugenol being the main compound with activity

against SE. They also observed an antimicrobial correlation with the presence of hydroxyl groups ($-\text{OH}$) in phenolic compounds and alcohols and highlighted that carvacrol and thymol showed higher antimicrobial activity compared to sulfanilamide. The limonene terpenoid from lemon EO at a concentration of 1.5% with 24 hours of exposure showed differences ($P<0.05$) when compared to any concentration at 1-hour and against the control at 24 hours in chicken meat. However, increasing the concentration of this EO in chicken meat seems to reduce its effectiveness against SE. In beef, this lemon EO at a concentration of 2% with 1-hour of exposure showed differences ($P<0.05$) when compared to 1.5%, 2.0%, and the control at 24 hours. In pork, its effect on SE was superior at 1.0% and 2.0% with 24 hours of exposure. The phenylpropene eugenol from clove EO at a concentration of 2.0% with 1-hour of exposure showed differences ($P<0.05$) when compared to any concentration at 24 hours and against 1.0% and the control at 1-hour in chicken meat. In beef, at a concentration of 1.5% with 24 hours of exposure, differences were identified when compared to 1.0%, 1.5%, and the control in the same type of meat. It also showed differences ($P<0.05$) when compared to any concentration in chicken meat at 1-hour and with all concentrations in chicken meat at 24 hours. Compounds such as eugenol (phenylpropene) have notable antimicrobial activity. Several authors mention that its antimicrobial effect is due to the interaction of $-\text{OH}$ groups with the cell membrane, causing the EOs to interact with the membrane lipids, altering permeability, integrity, osmoregulatory capacity, or structural functions. This leads to leakage of substances like potassium ions, a decrease in intracellular ATP, and an increase in extracellular ATP, ultimately causing cell lysis (Enayatifard *et al.*, 2021).

CONCLUSIONS

The effectiveness of essential oils on *Salmonella enterica* subsp. *enterica* serotype Enteritidis in different types of meat showed the greatest bacterial reduction in beef and with a 24 hour exposure time. The effectiveness of eucalyptus essential oil on bacterial growth was lower compared to oregano, clove, and lemon essential oils. Therefore, the terpenoid carvacrol, the phenylpropene eugenol, and the terpenoid

limonene showed a greater reduction in the survival of *Salmonella enterica* subsp. *enterica* serotype Enteritidis than 1,8-cineol from eucalyptus. In interactions between type of meat, exposure time, and type of essential oil, oregano essential oil showed no differences in chicken meat but did show differences in beef at 1% and 1-hour exposure when compared to any concentration in chicken or pork meat for both 1-hour and 24 hours exposures. Eucalyptus essential oil at 2%, 1-hour exposure in beef showed differences when compared to any concentration in chicken meat for both exposure times. Increasing the concentration of lemon essential oil in chicken meat seems to reduce its effectiveness against *Salmonella enterica* subsp. *enterica* serotype Enteritidis. Finally, clove essential oil showed the best results in beef, at 1.5% and with 24 hours of exposure. It is recommended to conduct an experiment testing a mixture of oregano and clove essential oils to enhance their bactericidal effect.

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