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FULL PAPER

Effect of essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), orange (*Citrus sinensis* var. Valencia) in the vapor phase on the antimicrobial and sensory properties of a meat emulsion inoculated with *Salmonella enterica*

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Abstract

The demand for healthier meat products is increasing remarkably and natural antimicrobial agents such as essential oils (EOs) are required, which can be applied in the vapor phase. For this experiment, the effectiveness of essential oils of oregano, thyme, and orange in the vapor phase on the microbiological and sensory characteristics of sausages inoculated with *Salmonella enterica* and stored at 4°C during 72 hrs and 144 hrs, were studied. Oregano EO with 2000 ppm was the most effective treatment against *Salmonella enterica* with a logarithmic reduction of 1.97 Log_{10} CFU/g compared to thyme 1.36 Log_{10} CFU/g and orange 1 Log_{10} CFU/g) after 144 hrs. In relation to the general acceptance level, the meat product exposed to the orange EO in vapor phase presented the highest approval by the judges nevertheless, however, were the ones that showed the least reduction in the microbial population. Finally, the results showed that the addition of essential oil in the vapor phase to meat products exerted a bactericidal effect with higher EOs concentrations and some also caused alterations in the sensorial properties of the product.

1. Introduction

According to the US Center for Disease Control and Prevention (CDC), millions of diseases caused by pathogens present in food originate every year (Ahmed and Shimamoto, 2014). Salmonellosis is one of the most reported zoonotic diseases. It is estimated that 95% of these infections are associated with food of animal origin (Hernandez *et al.*, 2005). Furthermore, some researchers have directly linked the consumption of contaminated meat with the appearance of outbreaks of salmonellosis (Torlak *et al.*, 2012).

In Mexico, the Institute of Epidemiological Diagnosis and Reference and the General Directorate of Epidemiology of the Ministry of Health, analyzed 24,394 *S. enterica* isolate recovered from human and non-human samples, which were isolated between the years 1972 and 1999 (Gutiérrez *et al.*, 2000). This work revealed that 4,926 (20.2%) isolates were recovered from food, and of these, 2,217 (9.1%) were obtained from meat and meat products. This information shows the potential risk of getting salmonellosis from consuming contaminated meat products.

The sausage occupies the first place in the intake in Mexico, according to the Mexican Meat Council (COMECARNE, 2018). Additionally, in recent years, it has been questioned the safety of using chemical additives, consumers increasingly demand the use of natural products as alternative preservatives in foods (Govaris *et al.*, 2010). The application of new and potential natural antimicrobial agents has increased significantly from different sources, such as microbial metabolites, plant extracts, and spices for food application (Cueva *et al.*, 2011).

The use of essential oils in the vapor phase to reduce foodborne pathogens and it has become a promising tool for food safety. The concentration of EOs necessary to inhibit/inactivate microorganisms can be minimized, and the antimicrobial effect can be ensured (Reyes *et al.*, 2019). Based on these considerations, the objective of the study was the evaluation of the antimicrobial

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capacity of the essential oils in the vapor phase from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and orange (*Citrus sinensis* var. Valencia), on a sausage inoculated with *Salmonella enterica*.

2. Materials and methods

2.1 Bacterial culture and inoculum preparation

A *Salmonella enterica* strain was previously isolated from food and identified by selective media such as Brilliant Green Agar and Bismuth Sulfite Agar and IMViC biochemical tests. Molecular analyses as 16S rRNA gene partial sequencing were used to confirm the identification of bacterial genera and species. Genomic DNA was obtained from culture sample (pellet of 500 mL) of *Salmonella* sp. (problem strain), grown in 5 mL of LB broth at 37°C with agitation, to an optical density 600 nm (OD600) of 0.5. The standard method of alkaline lysis was used to perform total DNA purification (Sambrook *et. al.,* 1989). Genomic DNA was used as a template for the 16S rRNA partial gene amplification by PCR using primers CD16F (5´-GGAGGCAGCAGTGGGGAATA-3´) and CD16R (5´-TGACGGGCGGTGTGTACAAG-3´) and a reaction mixture with Buffer 10X, 20 mM of $MgCl_2$, 10μM of dNTPs, 5U/μL of Dream Taq DNA polymerase and 10μM of oligonucleotides to a final volume of 200 μL. The PCR conditions were 94°C for 5 mins followed by 30 cycles of 94°C for 45 seg, 55°C for 50 seg and 72°C for 1 min and a final extension cycle of 72°C for 5 min.

The amplification product was 1062 bp and PCR products were purified using kit QIAquick (Qiagen, Germany) according to the manufacturer. Sequencing of PCR product was performed in Biomolecular Detection Center, BUAP (Puebla, Mexico). The 16S rRNA partial gene sequence was compared to sequences deposited in the GenBank database of the National Center for Biotechnology Information (NCBI), using the BLAST (Basic Local Alignment Search Tool) algorithm.

The microorganism was stored at -80°C grew with two consecutive transfers in tryptic soy broth (TSB; Difco, Becton Dickinson) and incubated at 37°C for 24 hrs. For the preparation of the bacterial inoculum, a population higher than 10^7 CFU/mL was assured, with the adjustment to an optical density (OD) of 1.1 to 620 nm. This was confirmed by counting in Trypticase Soy Agar medium (TSA; Bioxon, Mexico) plates incubated at 37°C for 24 hrs.

2.2 Sample inoculation

Meat sausages "Vienna" (Chimex® brand) were used, each piece had an average weight of 60 g and a length of 11 cm. The sausages were inoculated by immersion according to the procedure reported by Luna *et al.* (2015) with some modifications, the samples were introduced in a sterile Ziploc® bag with 50 mL of TSB inoculated with a population of 10^7 CFU/mL of *S. enterica*. Finally, the samples were constantly manual stirred for 1 min to ensure complete inoculation.

2.3 Antimicrobial treatments

2.3.1 Vegetables materials and essential oils (EOs) obtention

The oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and oranges (*Citrus sinensis* var. Valencia) were purchased from a local market in Puebla, Mexico. The spices (oregano and thyme), were used in their dehydrated form (moisture content approximately 11% DB) and the orange fruits were selected according to their index of maturity (with an approximate value of 12.23, MI (Madurity Index): °Brix/titratable acidity). The EOs were obtained according to the procedure reported by Masango (2005), using 200 g of thyme leaves, 250 g of oregano leaves, and 800 g of orange peels. They were subjected to distillation with a Clevenger type apparatus at different times: 110 mins, 75 mins, 138 mins with oregano, thyme, and orange, respectively.

2.3.2 Efficacy of antimicrobials in vapor phase

The antimicrobials agents were placed under a sterile glass plate with concentrations (0, 700, 1000, 1500 and 2000 mg of EO L^{-1}) and inoculated sausage pieces were introduced in a hermetically sealed plastic chamber (capacity of 1.8 L) (Figure 1). Samples chilled at 4±1 ° C were analyzed after 0, 72, and 144 hrs.

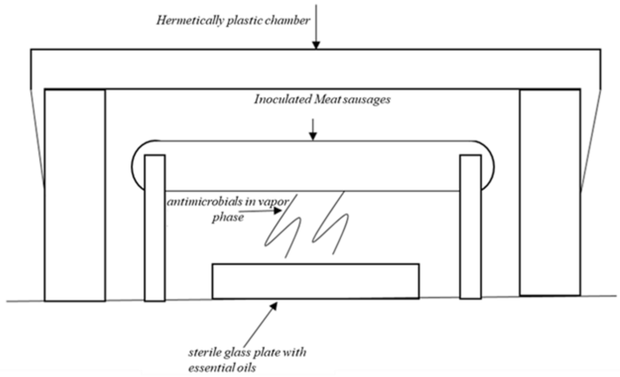

Figure 1. Design of the chamber for antimicrobial treatments with essential oils in vapor phase on emulsified products inoculated with *Salmonella enterica*

2.3.3 Microbiological analysis

After the storage times, the sausages (10 g) were removed aseptically from the chambers introduced in sterile stomacher (Mod 400 Interscience) bags with 90 mL of peptone water (0.1%) for homogenization for 1 min at high speed. The number of *S. enterica* cells were determined of serial dilutions which were spread on TSA plates and incubated at 35°C for 24 hrs. The identity of the colonies was confirmed with the classic IMViC biochemical tests and the kit proposed by Kim and Silva (2016), in which a change in color from green to orange confirmed a positive result. A parallel growth control was prepared to ensure that viable *S. enterica* cells and data were calculated as CFU because the entire sample was inoculated.

2.4 Sensory evaluation

Sensory analyzes were performed to know the level of acceptance and organoleptic alterations (color, aroma, taste and texture) of non-inoculated sausages exposed to the highest concentration (2000 of EO L^{-1}) with each of the essential oils at 144 h of storage. The sessions were realized with a panel of 50 untrained judges, and the 5-point hedonic scale was used (5 = I really like it, 4 = I like it, 3 = I like it / I don't like it, 2 = I dislike it and 1 = I dislike it a lot).

All determinations were made in triplicate, and the results of CFU/g were converted to Log_{10} values for data analysis. Logarithmic reductions were calculated by the difference between Log_{10} CFU/g control and Log_{10} CFU/g obtained of each treatment.

2.5 Data analysis

The results of the microbiological analyzes and the values of the sensory evaluations were analyzed by analysis of variance (ANOVA). The statistical program used was Minitab16®, considering 95% confidence, as a criterion of significant difference, between the samples. The significant differences between the average values were determined using the Tukey multiple comparison test.

3. Results and discussion

In this study, biochemical tests revealed that the Gram-negative bacteria isolated from food belonged to the genera *Salmonella* sp. Furthermore, the 16S rRNA partial gene sequence analysis of the strain confirmed for *Salmonella enterica* serovar Enteritidis. Figure 2 shows the behavior of *S. enterica* inoculated in chilled meats (common condition storage and marketing), this bacterium prevailed up to 144 hrs in samples of the control, which coincides with that reported by Rocatto *et al.* (2015) who consider that temperature is a decisive extrinsic factor for the development of this microorganism. Further, Helmuth *et al.* (2019) consider that the microbial growth in sausages is due to the fact that the product has not received a heat treatment such as boil and frying it before consumption, or in some cases the product has been exposed to cross-contamination.

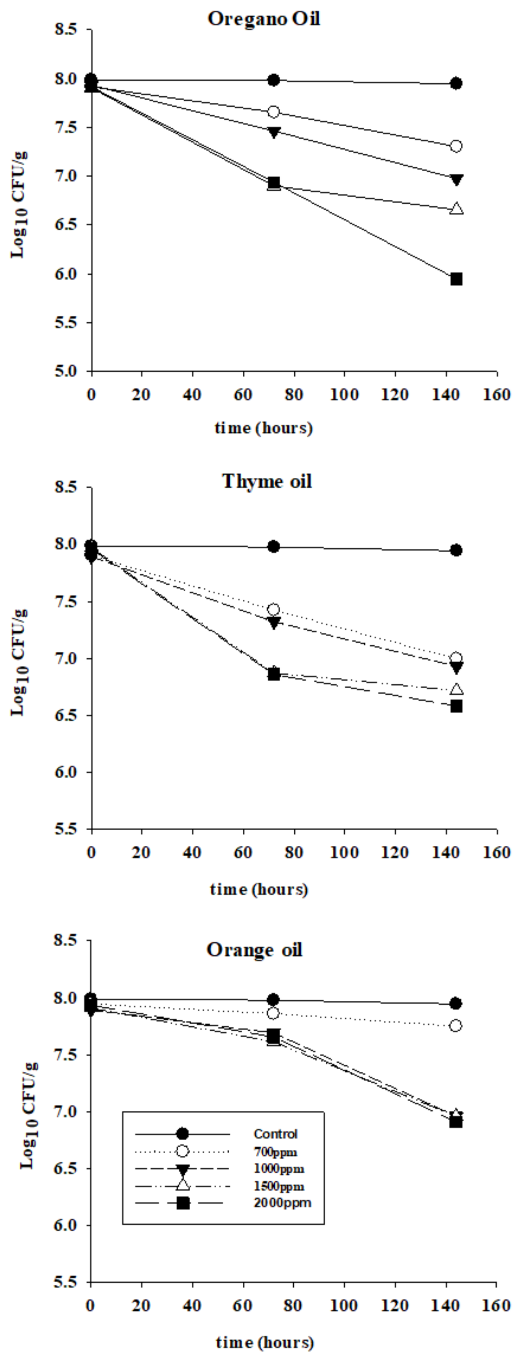

Figure 2. *Salmonella enterica* survival in emulsified meat product stored at 4°C for 72 and 144 hrs, exposed to the vapor phase of the essential oils of oregano, thyme and orange in concentrations of 0, 700, 1000, 1500 and 2000 mg de EOs L^{-1} of air

The previous trend agrees with the study carried out by Nedorostova *et al.* (2009) who mention that essential oils can increase their antimicrobial properties if they are applied in the vapor phase, presenting an inhibitory effect on food pathogens including *Salmonella enterica* serovar Enteritidis. Likewise, the above is related to that established by Ukuku and Sapers (2007) who confirm that the cooling conditions (4 to 5°C) reduce the

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volatilization rate of the EOs, favoring that the aromatic compounds come into contact with the entire surface of the inoculated product. Additionally, Gram-negative bacteria such as *Salmonella* are more susceptible to the effects of essential oils due to the hydrophobicity of their membrane, causing protons such as potassium ions to escape and inhibition in ATP synthesis (Fisher *et al.*, 2009; Tyagi and Malik, 2011).

According to a study by Matan *et al*. (2006), the antimicrobial effectiveness of EOs in the vapor phase depends on the composition of the atmosphere. While Mazzarrino *et al*. (2015), mention that oregano and thyme are more effective antimicrobial agents on microorganisms of importance in food such as *Salmonella* and *Listeria* compared to the effect of EOs from orange. In a particular way López *et al*. (2007), report that carvacrol present in oregano EO in the vapor phase, and exposure for 6 hrs inhibited 70% of the *Salmonella* population. Another report indicates that p-cymene, also contained in thyme, has activity on pathogens and spoilage microbiota present in meat (Lu and Wu, 2010).

The OE of oregano and thyme did not show a significant difference at concentration 1500 ppm with a storage time of 144 hrs, with logarithmic reductions 1.25 Log_{10} CFU/g and 1.22 log_{10} CFU/g, respectively. According to Oyedemi *et al.* (2009), the mechanisms of the action exerted by oregano oils are related to cell lysis by altering the lipid disposition of the membrane, with effects on its permeability.

Some studies, such as Lu and Wu (2010) demonstrated the inhibitory antimicrobial activity of thyme essential oil (200 ppm) achieved 4 Log reductions of *Salmonella*. While Nedorostova *et al.* (2009) confirmed the effectiveness of this spice on *S. enterica* ser. Enteritidis and *S. enterica* ser. Choleraesuis, the latter reported a 60% reduction in population when applying vapor phase treatments. In the investigation of Sartoratto *et al.* (2004) associated the antimicrobial effects of thyme with the contents of thymol, *α*-terpinene, and *p*-cymene.

Orange peel EO had the highest Log reductions with the higher concentrations (1500 and 2000 mg of EO L^{-1}) and 144 h of contact (Figure 2 and Table 1). These results correspond to those reported by the Tosun *et al.* (2018), who found inhibition of *Salmonella* until 96 hrs in inoculated salmon. According to Velázquez *et al*. (2013), the main compounds of the orange peel EO correspond to limonene, β-myrcene, β-pinene, α-pinene, as well as limonene representing 96.62%. Besides, Tyagi and Malik (2011) reported that monoterpenes concentrations in EOs are the main components with higher antimicrobial activity in the vapor phase than in direct contact and depend on their presence in gaseous form facilitating their solubilization in cell membranes. At the same time as Bajpai *et al*. (2012) confirmed that the limonene affects the cell membrane integrity inhibiting 90% *Salmonella* growth.

Table 1 shows the effect of EOs treatments in the vapor phase and contact times on the Log reductions of *S. enterica*. The most effective treatment on the *Salmonella enterica* population (1.97 Log_{10} CFU/g) was EOs of oregano with 2000 mg of EO L^{-1} compared to thyme (1.36 Log_{10} CFU/g) and orange (1 Log_{10} CFU/g).

Table 1. Logarithmic reductions of *Salmonella enterica* in emulsified meat product exposed to essential oils in the vapor phase stored at 4°C

Treatment		Storage time (hrs)	
Essential oil	Concentration (mg of EO L^{-1} of air)	72	144
Oregano	700	0.26 g K	0.61 e H
	1000	0.47 f J	0.96 d FG
	1500	1.01c E	1.25 b C
	2000	0.98 cd EF	1.97 a A
Thyme	700	0.55 g I	0.94 e FG
	1000	0.65 f H	1.01 d E
	1500	1.10 c D	1.22 b C
	2000	1.12 c D	1.36 a B
Orange	700	0.08 e M	0.19 d L
	1000	0.20 d L	0.93 b FG
	1500	0.29 c K	0.95 b FG
	2000	0.28 c K	1.01 a E

Logarithmic reductions represent the relationship between the average of log CFU/g of control (Log_{10} 7 CFU/g) and the Log_{10} CFU/g of the emulsified meat product exposed to each treatment. Different lowercase letters indicate significant differences ($p<0.05$) for the same essential oil treatment. Different capital letters indicate significant difference ($p<0.05$) among all treatments.

3.1 Effect of essential oils in the vapor phase in sensorial properties in sausages

The sensory attributes were evaluated at concentrations of 2000 mg of EOs L^{-1} of essential oil of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and orange peel (*Citrus sinensis* var. Valencia) by 144 hrs. The results of the sensorial analysis are presented in Table 2. The results were the average of scores which the examiners gave to each sample. Based on a 5-point hedonic scale, values more than three were considered organoleptically acceptable. As can be seen, when the attributes of aroma, texture, color, and flavor were evaluated, they did not present a significant difference ($P\geq0.05$). However, the attribute with the lowest values was the flavor of sausages treated with EOs of oregano

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Table 2. Sensory evaluation of sausage in contact with the vapor phase of different essential oils

Attribute	Orange (*Citrus sinensis* var. Valencia)	Oregano (*Origanum vulgare*)	Thyme (*Thymus vulgaris*)
Odor	3.70±1.13[a]	3.40±1.15[a]	3.67±0.82[a]
Texture	3.80±0.07[a]	3.75±0.83[a]	3.55±0.90[a]
Color	3.91±1.15[a]	4.22±0.69[a]	4.15±0.53[a]
Taste	3.17±1.03[a]	2.87±1.20[a]	2.62±1.25[a]
General acceptance	3.65±0.80[a]	3.25±1.05[ab]	2.97±1.18[b]
Final Score	3.61[a]	3.49[b]	3.39[c]

Different letters in the columns indicate significant difference ($p \leq 0.05$)

and thyme respectively. The application of larger amounts of essential oils could seriously interfere with the final organoleptic properties (Lis-Balchin *et al.*, 1998). In this study, a bactericidal effect was obtained at higher oil concentrations, although in this case, there were changes in taste parameters. According to Busatta *et al.* (2008), reducing the concentration of EOs would help to achieve a well-balanced product, satisfying the antimicrobial and acceptability aspects of consumer requirements. Finally, concerning the general acceptance, the EO in the orange vapor phase was the one that presented the highest approval by the judges. However, these meat products were the ones that showed the least reduction in the microbial population.

4. Conclusion

With the results shown in our study, it can be concluded that the EOs applied in the vapor phase significantly reduced the *Salmonella* population in sausages stored until 144 hrs under refrigerated conditions. Specifically, the highest reductions were presented with 1500 and 2000 mg of EO L^{-1} with the EO of oregano, while the results of the sensory analyzes with sausages exposed to the EO of orange (2000 mg of EO L^{-1}) indicated the highest values in the attributes of color, flavor and general acceptance. Finally, considering the EOs applied in the vapor phase results in a viable alternative to be used in the meat industry, it also represents an option to process waste such as orange peel to obtain natural antimicrobial agents with use-value in food.

Conflict of interest

The authors declare no conflict of interest.

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