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Efficacy of the combined application of oregano and rosemary essential oils for the control of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Enteritidis in leafy vegetables

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ABSTRACT

This study assessed the effect of the combined application of essential oils (EOs) from *Origanum vulgare* L. – oregano (OVEO) and *Rosmarinus officinalis* L. – rosemary (ROEO), alone or in combination at subinhibitory concentrations, against three pathogenic bacteria that are associated with fresh leafy vegetables: *Listeria monocytogenes* (*L. monocytogenes*), *Escherichia coli* (*E. coli*) and *Salmonella enterica* Serovar Enteritidis (*S.* Enteritidis). The inhibitory effects were evaluated by determining the minimum inhibitory concentration (MIC) and the fractional inhibitory concentration index (FICI) and assessing the viable cell counts in vegetable broth and artificially infected vegetables over time. Still, the effects of the EOs on native spoilage native flora were assessed. The MIC of OVEO was 0.6 μL/mL against the test strains either in single and mixed inoculum. The MIC of ROEO was 5 μL/mL against *L. monocytogenes* and *E. coli* and 10 μL/mL against *S.* Enteritidis in single inocula, whereas it was 10 μL/mL against the mixed inoculum. The FICI of the combined EOs was 0.5 against the mixed bacterial inoculum, which suggested a synergic interaction. The incorporation of OVEO and ROEO alone (MIC) or combined at different subinhibitory concentrations in vegetable broth resulted in a decrease in the viable cell counts of all test strains over 24 h. Similarly, the EOs alone or in the tested combinations reduced the viable cell counts of all test strains in experimentally infected fresh vegetables, besides to decrease the counts of spoiling native flora (mesophilic bacteria, enterobacteria and fungi). These findings reinforce the rational for the use of OVEO and ROEO in combination at subinhibitory concentrations to guarantee the safety and extend the shelf life of fresh vegetables.

1. Introduction

Demographic changes related to population aging and new life style trends have brought an increasing demand for ready-to-eat (RTE) foods. This demand has changed the status of foodborne diseases worldwide and had an important economic and social impact (Oliveira et al., 2015). In recent years, minimally processed vegetables (MPV) gained significant acceptance by consumers, which could be attributed to an increased consumer desire for fresh vegetables with reduced preparation times (Millan-Sango, McElhatton, & Valdramidis, 2015; Odumeru, Bouter, Knight, Lu, & Mckellar, 2002; Zhou et al., 2004). Prior to their sale, MPV are submitted to simple operations such as washing, peeling, slicing, shredding, sanitization, rinsing, drying and packaging to extend their shelf life and preserve their nutritive value while retaining the characteristics of fresh food (FDA, 2008).

Fresh vegetables are no longer considered a low-risk food in terms of safety. Fruits and vegetables can be contaminated with pathogens during all stages, from growth until consumption. The

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sources of contamination include the soil, manure (from humans and other animals), water, insects, post-harvest handling, washing, cutting and transportation (Beuchat, 2002; Bhagwat & Matthews, 2006). Sanitization is crucial to decrease the occurrence of microbial hazards in MPV because minimal processing is not an end-point preservation treatment (Sagoo, Little, Ward, Gillespie, & Mitchell, 2003). The increased association of MPV with foodborne outbreaks has intensified consumers' concerns regarding the safety of these products (Sant'ana et al., 2012). Among the classical pathogens that are considered threats to the safety of MPVs, *Salmonella* spp. and pathogenic *Escherichia coli* have been particularly concerning (CDC, 2013); however, emerging pathogens, such as *Listeria monocytogenes*, have more recently been linked to outbreaks associated with the consumption of fresh vegetables worldwide (Ethelberg et al., 2010; Friesema et al., 2007, 2008; Johnsen, Lingaas, Torfoss, Strøm, & Nordøy, 2010; Sagoo et al., 2003). The decontamination methods used for vegetables aim to reduce the microbial populations of the processing system without necessarily eliminating them. A variety of disinfectants, including chlorine, hydrogen peroxide, organic acids and ozone, have been used to reduce the initial bacterial populations on vegetables (Beuchat, 1998; Gil, Selma, López-Galvez, & Allende, 2009; Suwa, Oie, & Furukawa, 2013). Chlorine and chlorine-based compounds, such as hypochlorite, are probably the most widely used sanitizer for the treatment of fresh vegetables. However, some studies have shown that the chlorine concentrations (50–200 ppm) that are traditionally used for decontamination are not effective in successfully reducing pathogen loads on vegetables (Behrsing, Winkler, Franz, & Premier, 2000; Delaquis, Stewart, Cazaux, & Toivonen, 2002; Lee & Baek, 2008). Considering that fresh vegetables have the potential to harbor pathogenic bacteria, the development of new and effective sanitizing procedures has received much attention (Azerêdo et al., 2011; Oliveira et al., 2015; Sousa et al., 2013).

There has been increasing pressure to replace chemically synthesized antimicrobials with natural alternatives in the food industry (Xu et al., 2007). This pressure has led to a particularly increased interest in the use of essential oils (EOs) as natural sanitizers for fresh vegetables. EOs from *Origanum vulgare* L. – oregano (OVEO) and *Rosmarinus officinalis* L. – rosemary (ROEO) have been found to be effective in inhibiting a variety of bacteria, including those that contaminate RTE vegetables (Azerêdo, Figueiredo, Souza, & Stamford, 2012; Sousa et al., 2012). Many EOs and their individual constituents are considered to be 'Generally Recognized as Safe' (GRAS) at the doses typically used in foods (Burt, 2004) and have been approved by the Food and Drug Administration (FDA) for use in edible products (FDA, 2002). Additionally to improve the microbial safety, EOs should not negatively impact the sensory aspects of fresh vegetables influencing their acceptance by consumers (Azerêdo et al., 2012). Sometimes, the required amounts of EOs to establish the desired antimicrobial effects in foods can result in odors and flavors that are unpleasant to the consumer (Gutierrez et al., 2009). The addition of small amounts of EOs in mixtures may be a way to provide the balance between sensory acceptability and antimicrobial efficacy because the combined action of these substances, even in subinhibitory quantities, may potentiate their antimicrobial efficacy (Sousa et al., 2012).

Therefore, this study was performed to assess the inhibitory effects of OVEO and ROEO alone or in combination against a mixed culture of bacteria associated with the contamination of fresh leafy vegetables, namely *E. coli*, *L. monocytogenes* and *Salmonella enterica* Serovar Enteritidis (*Salmonella* Enteritidis). The effects were measured by determining the minimum inhibitory concentration and fractional inhibitory concentration index and assessing cell viability in vegetable broth and in artificially infected fresh leafy vegetables over time. Additionally, the effects of the EOs on spoilage native flora in fresh leafy vegetables were assessed.

2. Material and methods

2.1. Material

The OVEO (batch ORETU679; density at 20 °C: 0.90; refractive index at 20 °C: 1.49) and ROEO (batch ROSTUN04; density at 20 °C: 0.94; refractive index at 20 °C: 1.51) were purchased from Laszlo Aromaterapia Indústria e Comércio Ltda. (Minas Gerais, Brasil). Their quality parameters are described in an accompanying technical report. This supplier extracts essential oils on an industrial scale by steam distillation. The EOs were assayed at concentrations ranging from 80 to 0.03 μL/mL. EO emulsions were prepared in brain-heart infusion (BHI) broth (Himedia, India) using bacteriological agar (1.5 g/L) as a stabilizing agent (de Souza, Oliveira, Conceicao, & Barros, 2010).

L. monocytogenes ATCC 7644, *E. coli* UFEPEDA 224 and *Salmonella* Enteritidis UFEPEDA 414 used as test microorganisms were gently provided by the Microorganism Collection, Department of Antibiotics, Federal University of Pernambuco (Recife, Brazil). An inoculum of each test bacterium was obtained by preparing suspensions in sterile saline solution (NaCl 0.85% p/v) from overnight cultures grown in BHI agar at 37 °C. Each strain was grown in BHI broth at 37 °C for 18 h (late exponential growth phase), harvested by centrifugation (4500 *g*, 15 min, 4 °C), washed twice in sterile PBS and re-suspended in sterile PBS to obtain standard cell suspensions, for which the OD reading at 625 nm (OD_{625}) was 0.1, to provide viable cell counts of approximately 8 log colony forming unit per milliliters – CFU/mL (McMahon et al., 2008). A mixed inoculum was obtained by mixing the different bacterial suspensions at a ratio of 1:1:1. This level of inoculum was used because vegetable decontamination studies require high numbers of cells in the inoculum to enable the measurement of several log reductions in colony forming unities per gram – CFU/g (Beuchat et al., 2001).

Time-kill assays were performed using a vegetable broth composed of iceberg lettuce (*Lactuca sativa* L.) and chard (*Beta vulgaris* L. var. cicla) as the substrates for bacterial cultivation. The leafy vegetables were purchased from a local wholesale market in João Pessoa (Brazil) on the day of harvest and transported for less than 30 min under refrigerated conditions. A mixture (1:1) of the samples containing 60 g of each leafy vegetable was mashed with 400 mL of distilled water using a domestic blender. The mixture was then vacuum filtered using Whatman no. 1 filter paper. The obtained material was sterilized by filtration using a Millipore 0.22 μm filter unit (Azerêdo et al., 2011). The filtered broth was stored at −20 °C in 50-mL aliquots, and when required, an aliquot was thawed under refrigeration (7 ± 1 °C) and used for the assays.

2.2. Identification of the EO constituents

The constituents of OVEO and ROEO were identified by gas chromatography coupled with mass spectrometry – GC–MS (CGMS-QP2010 Ultra Shimadzu, Kyoto, Japan). GC–MS analysis was performed under the following conditions: a RTX-5MS capillary column (30 m × 0.25 mm × 0.25 μm); temperature programing: 60–240 °C (3 °C/min); injector temperature: 250 °C; detector temperature: 220 °C; carrier gas: helium adjusted to 0.99 mL/min speed; ionizing energy: 70 eV; mass range (*m*/*z*): 40–500. To identify the individual EO constituents, the spectra bank of the GC/MS, NIST/EPA/NIH Mass Spectral Database (Version 1.7) was used. The quantification of the constituents was obtained by normalizing the areas of each detected constituent and expressed the result in terms of the percentage area (%).

2.3. Determination of the minimum inhibitory concentration (MIC)

The MIC values of OVEO and ROEO against a single and mixed inoculum of the test strains were determined using macrodilution in broth (Nostro et al., 2001). Four milliliters of double strength BHI broth (Himedia, India) was inoculated with 1 mL of bacterial inoculum (single or mixed), mixed with 5 mL of the solution of OVEO or ROEO and vigorously mixed for 30 s using a vortex. The system was statically incubated for 24 h at 37 °C. The MIC was defined as the lowest concentration of each EO required to prevent visible bacterial growth (Nostro et al., 2001). Control flasks without the EOs were tested in a similar process.

2.4. Determination of the fractional inhibitory concentration index (FICI)

The checkerboard method was performed using macrodilution in broth to obtain the FICI for the combined application of OVEO and ROEO using a mixed inoculum of the test strains (Oliveira et al., 2015). The FICI, which is characterized as a simple mathematical approach to quantitatively describe interactions (Iten, Saller, Abel, & Reichling, 2009), was calculated as follows:

$$\frac{\text{MIC of OVEO in combination with ROEO}}{\text{MIC of OVEO alone}} + \frac{\text{MIC of ROEO in combination with OVEO}}{\text{MIC of ROE alone}}$$

The OVEO and ROEO were assayed at the MIC × 2, MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC (OVEO: 1.2, 0.6, 0.3, 0.1, 0.05, 0.02 μL/mL; ROEO: 20, 10, 5, 2.5, 1.2 and 0.6 μL/mL) alone and with different combinations of each different concentration of each EO. The results were interpreted as synergy (FIC ≤ 0.5), addition (0.5 ≤ FIC ≤ 1), indifference (1 ≤ FIC ≤ 4) and antagonism (FIC > 4) (Gutierrez, Barry-Ryan, & Bourke, 2008; Schelz, Molnar, & Hohmann, 2006).

2.5. Effects of the tested EOs on the survival of bacteria in vegetable broth

The effect of EOs alone (MIC) and in combination (1/2 MIC OVEO + 1/2 MIC ROEO; 1/2 MIC OVEO + 1/4 MIC ROEO; 1/4 MIC OVEO+ 1/2 MIC ROEO; 1/4 MIC OVEO + 1/4 MIC ROEO) (Table 1) on the survival of bacterial strains in mixed inoculum in vegetable broth was evaluated using the viable cell count procedure. For this test, 4 mL of vegetable broth was inoculated with 1 mL of the bacterial inoculum. Then, 5 mL of the single or combined EO solutions was added to the system and gently shaken for 30 s to produce a final viable cell count of each bacteria of approx. 7 log CFU/mL. The system was incubated at 7 °C. At different time intervals (2, 4, 8, 12 and 24 h), 1 mL of the suspension was serially diluted (10^{-1} – 10^{-5}) in a sterile saline solution (NaCl 0.85 g/100 mL) and inoculated onto media selective for each bacteria. The selective media were *Listeria* selective agar + Listeria Selective Supplement II (Himedia, India) for *L. monocytogenes*, Eosyne-Metilen-Blue (EMB) agar (Himedia, India) for *E. coli* and *Salmonella – Shigella* agar (Himedia, India) for *S.* Enteritidis for 24–48 h at 37 °C (Azêredo et al., 2011; Sousa et al., 2012). Control flasks without the EOs were similarly tested. The plates inoculated with aliquots collected from broth containing the EOs were always incubated for 24 h longer at adequate temperature than were those collected from the control systems. The results were expressed as the reduction in bacterial counts (log CFU/mL reduction cycles) in relation to the initial bacterial population – CFU/mL at time zero (log N_0–N; where N_0 was the initial count at time zero and N was the count after incubation for each indicated time at 37 °C).

Table 1
Different concentrations of the essential oils from *O. vulgare* L. (OVEO) and *R. officinalis* L. (ROEO) used alone and/or in combination in assays of microbial survival in vegetable broth and/or in leafy fresh vegetables.

Tested concentrations		
OVEO	Alone or combined with	ROEO
MIC: 0.6 μL/mL	alone	–
–	alone	MIC: 10 μL/mL
1/2 MIC: 0.3 μL/mL	combined with	1/2 MIC: 5 μL/mL
1/2 MIC: 0.3 μL/mL	combined with	1/4 MIC: 2.5 μL/mL
1/4 MIC: 0.15 μL/mL	combined with	1/2 MIC: 5 μL/mL
1/4 MIC: 0.15 μL/mL	combined with	1/4 MIC: 2.5 μL/mL

MIC: Minimum Inhibitory Concentration; (–): not tested.

2.6. Effects of the EOs on bacterial survival in fresh vegetables

The effect of the EOs alone (MIC) or in combination (1/2 MIC OVEO + 1/2 MIC ROEO; 1/2 MIC OVEO + 1/4 MIC ROEO) (Table 1) on the survival of the bacterial strains in the mixed inoculum on fresh vegetables was evaluated using the viable cells count procedure. For this procedure, portions (90 g) of a pool of iceberg lettuce and chard (at a rate of 1:1) that were previously washed with sterile distilled water were shredded aseptically and inoculated with the bacteria according to the following procedure: the vegetable sample were submerged in 900 mL of mixed inoculum, gently rotated with a sterile glass stem for 5 min to ensure effective inoculation and air-dried for 1 h in a bio-safety cabinet. Subsequently, the vegetables were submerged in 250 mL of OVEO or ROEO solutions either alone or in combination for 5 or 10 min at 25 °C. Then, a 25-g sample of the vegetables was aseptically obtained, transferred into a sterile stomacher bag containing 225 mL of sterile saline solution (0.85 g/100 mL) and homogenized for 60 s. Subsequently, a decimal dilution was made in the same diluent, and bacterial counting was performed by spread-plating 0.1 mL of the appropriate sample dilution on sterile selective agar (*Listeria* selective agar + Listeria Selective Supplement II (Himedia, India) for *L. monocytogenes*, EMB agar for *E. coli* (Himedia, India) and *Salmonella – Shigella* agar (Himedia, India) for *S.* Enteritidis (Azêredo et al., 2011; Sousa et al., 2012) for 24–48 h at 37 °C. Control flasks containing sterile distilled water were tested in the same way. Plates inoculated with aliquots collected from systems containing the tested EOs were always incubated for 24 h longer at adequate temperatures than were those collected from control systems. The results were expressed as the reduction in bacterial counts (log CFU/mL reduction cycles) in relation to the initial bacterial population – CFU/mL at time zero (log N_0–N; where N_0 was the initial count at time zero and N was the count after incubation for each indicated time at 37 °C).

2.7. Effect of EOs on survival of spoilage native flora in fresh vegetables

Portions of 90 g of iceberg lettuce and chard (in a rate of 1:1) were shredded by glove-covered hands and immediately submerged in 250 mL of the solutions of OVEO and ROEO alone (MIC) or in mixture (1/2 MIC OVEO + 1/2 MIC ROEO, 1/2 MIC OVEO + 1/4 MIC ROEO) (Table 1) and softly rotated for 5 min or 10 min at 28 °C using a sterile glass stem to ensure complete coverage and contact of surfaces with the EOs solutions. Subsequently, a 25 g sample of the vegetables was aseptically taken, transferred into a sterile stomacher bag containing 225 mL of sterile peptone water (1 g/L), and homogenized for 60 s. Then, a decimal dilution series (10^{-2} – 10^{-5}) was made in the same diluent, and count of the native spoilage flora was performed by pour-plating 1 mL of the

appropriate sample dilutions on Plate Count Agar (Himedia, India) at 37 °C (24–48 h) for total mesophilic bacteria and at 6 °C (7 d) for psychotrophic bacteria, and by spread-plating 0.1 mL onto Eosyne-Metilen-Blue agar (Himedia, India) at 37 °C (24 h) for *Enterobacteriaceae* and Potato Dextrose agar (Himedia, India) with pH adjusted to 3.5 using tartaric acid (1%) at 28 °C (48–72 h) for fungi. The results were expressed in the log of cfu/mL (Sousa et al., 2012). Control flasks containing sterile distilled water were tested in the same way. Plates inoculated with aliquots collected from systems containing the tested EOs were always incubated for 24 h longer at adequate temperatures than were those collected from control systems. The results were expressed as the reduction in microbial counts (log CFU/mL reduction cycles) (log Nc − Nt; where Nc was the count found in non-treated (control) leafy vegetables and Nt was the count found in leafy vegetables treated with the EOs alone or in combinations for each indicated exposure times at appropriate temperature).

The detection limit of the viable cell detection was 2 log cfu/mL for all experiments.

2.8. Reproducibility and statistics

All assays were performed in triplicate in three independent experiments, and the results were expressed as an average of the assays. Statistical analysis was performed to determine significant differences ($p < 0.05$) using ANOVA followed by a post hoc Tukey test in the Sigma Stat 3.5 software (Jandel Scientific Software, San Jose, California).

3. Results

3.1. Identification of the EO constituents

As shown in Table 2, the GC–MS analysis identified six compounds at amounts greater than 1% in both OVEO and ROEO (Table 2). Thymol (69.3%) was the most prevalent compound in OVEO, followed by *p*-cymene (13.1%) and γ-terpinene (6.01%). The compounds myrcene (1.1%), linalool (2.71%) and α-pinene (1.6%) were found in minor amounts. For ROEO, the compounds detected at higher amounts were eucalyptol (35.75%), camphor (28.7%) and limonene (24.88%). Other compounds (viz. α-pinene, *p*-cymene and γ-terpinene) were found in the range of 1.29–1.97 % in ROEO.

3.2. MIC and FICI values

The MIC value obtained for OVEO was 0.6 μL/mL against *L. monocytogenes* ATCC 7644 (*L. monocytogenes*), *E. coli* UFEPEDA 224 (*E. coli*) and *S.* Enteritidis UFEPEDA 414 (*S.* Enteritidis) either in the single or mixed inoculum (Table 3). The MIC value of ROEO was 5 μL/mL against *L. monocytogenes* and *E. coli* and 10 μL/mL against *S.* Enteritidis in single inoculum. The MIC value of ROEO was 10 μL/mL against the mixed inoculum. The FICI for the combined application of OVEO and ROEO against the mixed inoculum was 0.5, which suggested a synergistic interaction. The OVEO and ROEO inhibited the bacterial growth when tested up to a combination of 1/4 MIC +1/4 MIC, respectively. The test strains had the ability to grow at the tested subinhibitory concentrations of both OVEO and ROEO when applied alone (data not shown).

Table 2
GC–MS analysis of the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO).

Constituents[a]	Percent of essential oil total mass
	OVEO
thymol	69.30
p-cymene	13.10
γ-terpinene	6.01
myrcene	1.10
linalool	2.71
α-pinene	1.60
	ROEO
eucalyptol	35.75
camphor	28.7
limonene	24.88
α-pinene	1.97
p-cymene	1.67
γ-terpinene	1.29

[a] Constituents detected in amounts >1%.

3.3. Effects on survival of bacteria in vegetable broth

The effects of OVEO and ROEO alone at the MIC or in different combinations of 1/4 MIC and 1/2 MIC on the survival of a mixed population of *L. monocytogenes*, *E. coli* and *S.* Enteritidis cultivated in vegetable broth were assessed over 24 h. A sharp drop ($p < 0.05$) in the viable cell counts of all tested strains was observed over the assessed time intervals when either OVEO or ROEO was incorporated into the vegetable broth, with the exception of *S.* Enteritidis exposed to ROEO at its MIC (Fig. 1A – 1C). The detection of ≥3 log CFU/mL cycles (≥3 log cycles) – decrease (>99.99% reduction) in the initial viable cell counts of *L. monocytogenes* and *E. coli* exposed to OVEO at MIC occurred as early as 2 and 4 h of exposure, respectively. When ROEO was assayed at its MIC, a decrease of ≥3 log cycles in the viable counts of *L. monocytogenes* and *E. coli* was observed after 4 and 8 h, respectively (Fig. 1A–B). For *S.* Enteritidis, a ≥3 log cycles – decrease in the viable cell counts was observed after 8 h in the OVEO-treated cells. Cells of *S.* Enteritidis treated with ROEO at MIC were found to have a smaller decrease (up to 1.7 log cycles; > 90% reduction) in the viable counts early in the assessed time intervals (after 1–4 h of exposure). However, a slight and linear recovery was in the cell counts was observed later during the assessed time intervals (Fig. 1C).

The incorporation of OVEO and ROEO in vegetable broth in combinations of 1/2 MIC OVEO +1/2 MIC ROEO, 1/2 MIC OVEO +1/4 MIC ROEO and 1/4 MIC OVEO +1/2 MIC ROEO resulted in a ≥3 log cycles – decrease in the initial viable counts of *L. monocytogenes* and *E. coli*, although the time required to establish this decrease varied according to the target strain and the amount of combined EOs. The combination of 1/2 MIC OVEO +1/2 MIC ROEO caused similar reductions in the *L. monocytogenes* and *E. coli* viable counts after 4 and 8 h of exposure, respectively. For the combination of 1/4 MIC OVEO +1/2 MIC ROEO, this reduction was noted after 24 h in both strains. The decrease in the viable counts of *E. coli* and *L. monocytogenes* treated with 1/4 MIC OVEO +1/4 MIC ROEO were near to 2.5 log cycles (>99.9% reduction) after 24 h of exposure. Only the combination of 1/2 MIC OVEO +1/2 MIC ROEO could cause a ≥3 log cycles – decrease in the *S.* Enteritidis counts. The other tested combinations caused decreases in the range of 1.5–2.7 log

Table 3
Minimum inhibitory concentration (MIC) of the essential oils from *O. vulgare* L. (OVEO) and *R. officinalis* L. (ROEO) against *L. monocytogenes*, *E. coli* and *S.* Enteritidis in single and mixed inoculum.

Strains	MIC values (μL/mL)	
	OVEO	ROEO
Single inoculum		
L. monocytogenes ATCC 7644	0.6	5
E. coli UFEPEDA 224	0.6	5
S. Enteritidis UFEPEDA 414	0.6	10
Mixed inoculum	0.6	10

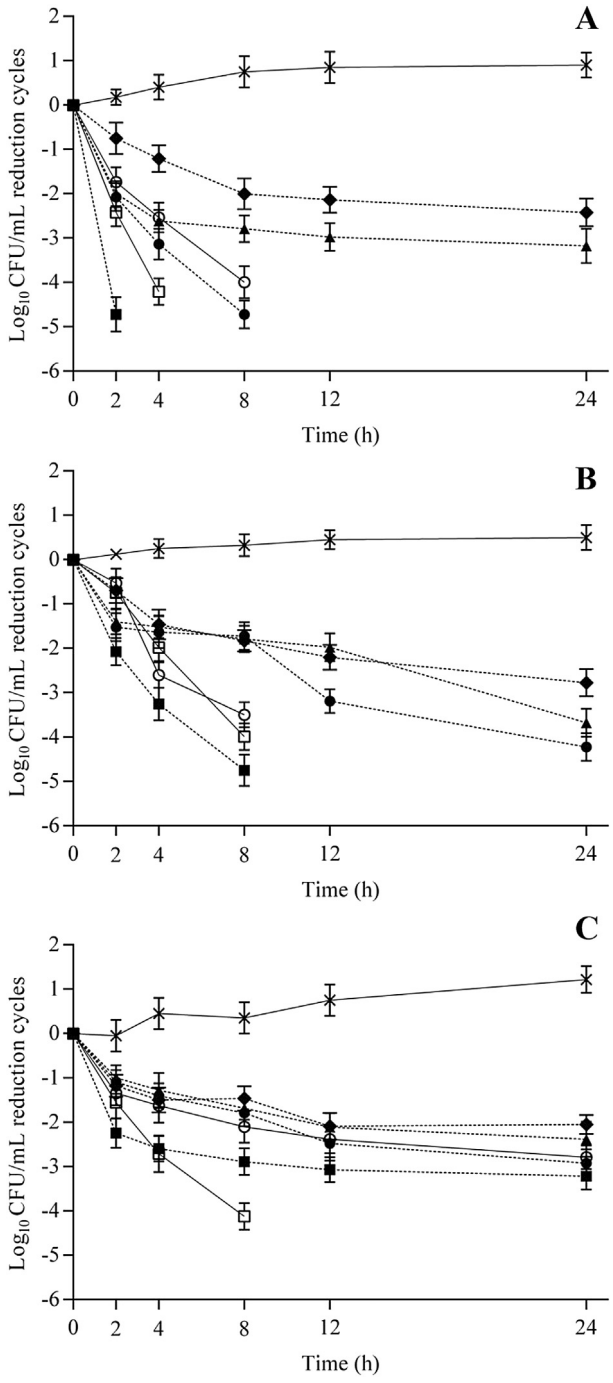

Fig. 1. Reduction cycles (Log_{10} CFU/mL) of the initial viable cell counts of *L. monocytogenes* ATCC 7644 (A), *E. coli* UFEPEDA 224 (B) and *S.* Enteritidis UFEPEDA 414 (C) in vegetable broth (7 °C) as a function of different concentrations of the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. over 24 h (ROEO) alone or in combination. (+) Control; (●) MIC OVEO: 0.6 μL/mL; (■): MIC ROEO: 10 μL/mL; (○): 1/2 MIC OVEO: 0.3 μL/mL + 1/2 MIC ROEO 5 μL/mL; (□): 1/2 MIC OVEO: 0.3 μL/mL + 1/4 MIC ROEO: 2.5 μL/mL; (△): 1/4 MIC OVEO: 0.15 μL/mL + 1/2 MIC ROEO: 5 μl/mL; (*): 1/4 MIC OVEO: 0.15 μL/mL + 1/4 MIC ROEO: 2.5 μL/mL.

cycles (>90% to > 99.9% reduction) at the end of the assessed exposure time. Over the evaluated time interval, the systems containing OVEO and/or ROEO showed lower ($p < 0.05$) viable cell counts than the control systems.

3.4. Effects on the survival of bacteria on artificially infected fresh leafy vegetables

Regarding the results obtained in the vegetable broth, the combinations of 1/4 MIC OVEO +1/2 MIC ROEO and 1/4 MIC OVEO +1/4 MIC ROEO were not assayed in fresh leafy vegetables because they did not cause a ≥3 log cycles-decrease in the viable cell counts over the assessed time period or because the time periods required to establish this effect were longer than those observed for the other combinations tested.

The application of OVEO and ROEO to artificially infected fresh leafy vegetables, either alone or in combination at different subinhibitory concentrations, caused a reduction ($p < 0.05$) in the initial viable cell counts of all assayed strains in mixed inoculum (Fig. 2A–F). After a 5-min treatment, only OVEO at its MIC caused a ≥3 log cycles-decrease in the initial counts of all strains. The reductions caused by the other treatments were in a range of 1.5–2.8 log cycles (>90% to > 99.9%). After 10 min of treatment, either OVEO or ROEO at their respective MICs or the combination 1/2 MIC OVEO +1/2 MIC ROEO caused a similar decrease in the viable cell counts of *L. monocytogenes* and *E. coli* (Fig. 2C and E). The decrease in the viable counts of *S.* Enteritidis exposed to ROEO alone or to the tested combinations was in the range of 1.5–2.5 log cycles (Fig. 2E and F). Overall, the exposure of the EOs for 10 min caused a greater decrease ($p < 0.05$) in the viable cell counts in comparison to 5 min. Viable cell counts in the control systems (5 min and 10 min) were always close to 7 log CFU/mL, and these counts were higher ($p < 0.05$) than those found when the strains were treated with OVEO and/or ROEO.

In both assays using vegetable broth and fresh vegetables, *L. monocytogenes* was found to be most sensitive to the EOs alone or in combination, whereas *S.* Enteritidis was found to be the least sensitive. Furthermore, the inhibitory effects of the EOs occurred in a time-dependent manner.

3.5. Effects on survival of spoiling native flora on fresh leafy vegetables

The application of OVEO and ROEO to fresh leafy vegetables, either alone or in combination at different subinhibitory concentrations, caused a reduction ($p < 0.05$) in viable counts of native spoilage flora in comparison to the control assay (Fig. 3A–F). After a 5-min treatment, the OVEO and ROEO at their respective MICs and the combination 1/2 MIC OVEO +1/2 MIC ROEO caused the greatest ($p < 0.05$) decreases in the counts of mesophilic bacteria (1.6–2.5 log cycles) and enterobacteria (1.7–2.7 log cycles). The decrease in mold counts (1.9–2.2 log cycles) were similar ($p > 0.05$) in leafy vegetables treated with either each EO alone at its MIC or in combination of 1/2 MIC OVEO + 1/2 MIC ROEO or 1/4 MIC OVEO + 1/2 MIC ROEO. After a 10-min treatment, the OVEO and ROEO, either alone or in combination at different subinhibitory concentrations, caused similar decrease ($p > 0.05$) in the counts of all assessed groups (or family) of spoilage microorganisms. The decrease in counts of mesophilic bacteria, enterobacteria and fungi was always close to 3.0, 2.5 and 3.1 log cycles, respectively. Overall, the exposure of the EOs for 10 min caused a greater decrease ($p < 0.05$) in spoilage native flora counts in comparison to 5 min.

The counts of mesophilic bacteria, enterobacteria and fungi in control leafy vegetables after a 5- and 10-min treatment were 5.6 and 5.9 CFU/g, 5 and 4.8 CFU/g and 5.2 and 5.3 CFU/g, respectively. No counts of psychotrophic bacteria were found in both control and treated leafy vegetables (data not show).

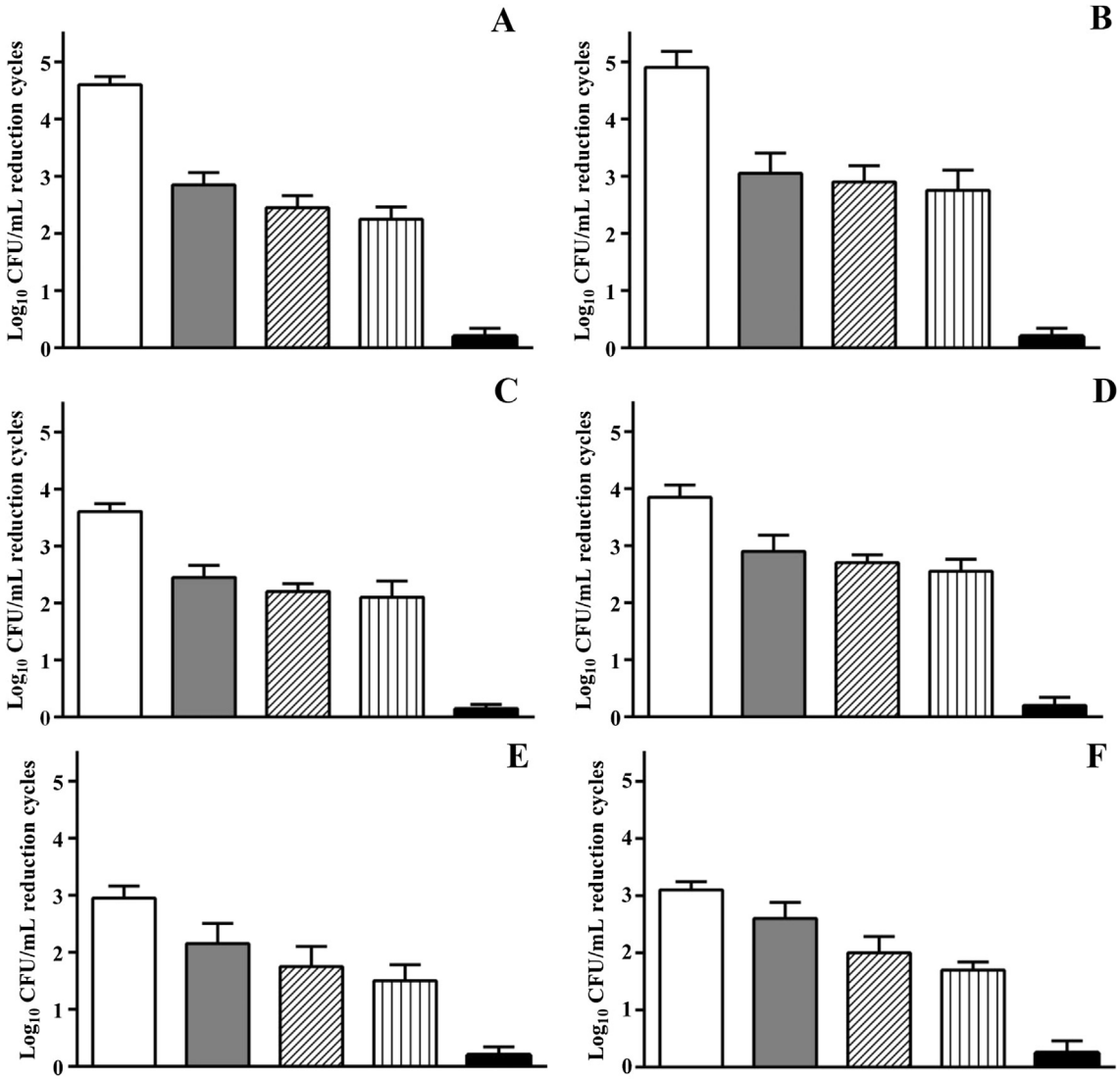

Fig. 2. Reduction cycles (Log_{10} CFU/mL) of the initial viable cell counts of *L. monocytogenes* ATCC 7644 (A–B), *E. coli* UFEPEDA 224 (C–D) and *S.* Enteritidis UFEPEDA 414 (E–F) in fresh leafy vegetables (28 °C) as a function of different concentrations of the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO) alone or in combination, after a 5-min (A, C, E) and 10-min (B, D, F) treatment (white bars: reduction caused by OVEO at its MIC: 0.6 μL/mL; grey bars: reduction caused by ROEO at its MIC: 10 μL/mL; diagonal dashed bars: reduction caused by 1/2 MIC OVEO: 0.3 μL/mL + 1/2 MIC ROEO: 5 μL/mL; vertical dashed bars: reduction caused by at 1/2 MIC OVEO: 0.3 μL/mL + 1/4 ROEO: 2.5 μL/mL); black bars: reductions observed in control assays: OVEO: 0 μL/mL + ROEO: 0 μL/mL.

4. Discussion

This study showed the inhibitory effects of OVEO and ROEO alone at MIC or in combination at different subinhibitory concentrations on the survival of *L. monocytogenes*, *E. coli* and *S.* Enteritidis in single or mixed inocula. These results are interesting because studies testing the effects of EOs (or their individual constituents) on pathogenic or spoiling bacteria in mixed culture are still scarce. The low available number of studies with candidate compounds for use as antimicrobials in foods using mixed populations of target bacteria is somewhat conflicting because the use of multispecies inocula (or even multi-strain inocula) has been recommended in studies that have used specifically defined conditions that limit the growth or survival of the bacterial species (Romero, Pinto, Patriarca, & Vaamonde, 2010). Some researchers have stated that the response of mixed cultures of bacteria to challenge with EOs or their individual constituents could provide more realistic information regarding the antimicrobial efficacy of these compounds on food substrates (Kurekci et al., 2013; Oliveira et al., 2015).

OVEO showed stronger inhibitory effects against all test bacteria in both single and mixed inocula than did ROEO, based on their respective MIC values (the MIC of OVEO was 16-fold lower than that of ROEO). *S.* Enteritidis was most tolerant to ROEO, and the MIC value against this bacterium was one-fold greater than the MIC values against the other test bacteria. Still, this greater tolerance of *S.* Enteritidis may have had some influence on the higher MIC value (a one-fold increase) observed in ROEO against the bacterial strains in the mixed inoculum. The low observed MIC value of OVEO against the test strains (<1 mg/mL or μL/mL, Van Vuuren, 2008) reinforced the noteworthy antibacterial effects of this EO, in contrast to the high MIC value showed by ROEO. The difference in the inhibitory effects of OVEO and ROEO (considering their often-detected MIC values) has been related to their particular profile of major constituents (Carović-Stanko et al., 2010). An approximate general ranking of the individual constituents of the EOs possessing the highest antimicrobial effects is as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons (Ballester-Costa, Sendra, Fernández-López, Pérez-Álvarez, & Viuda-

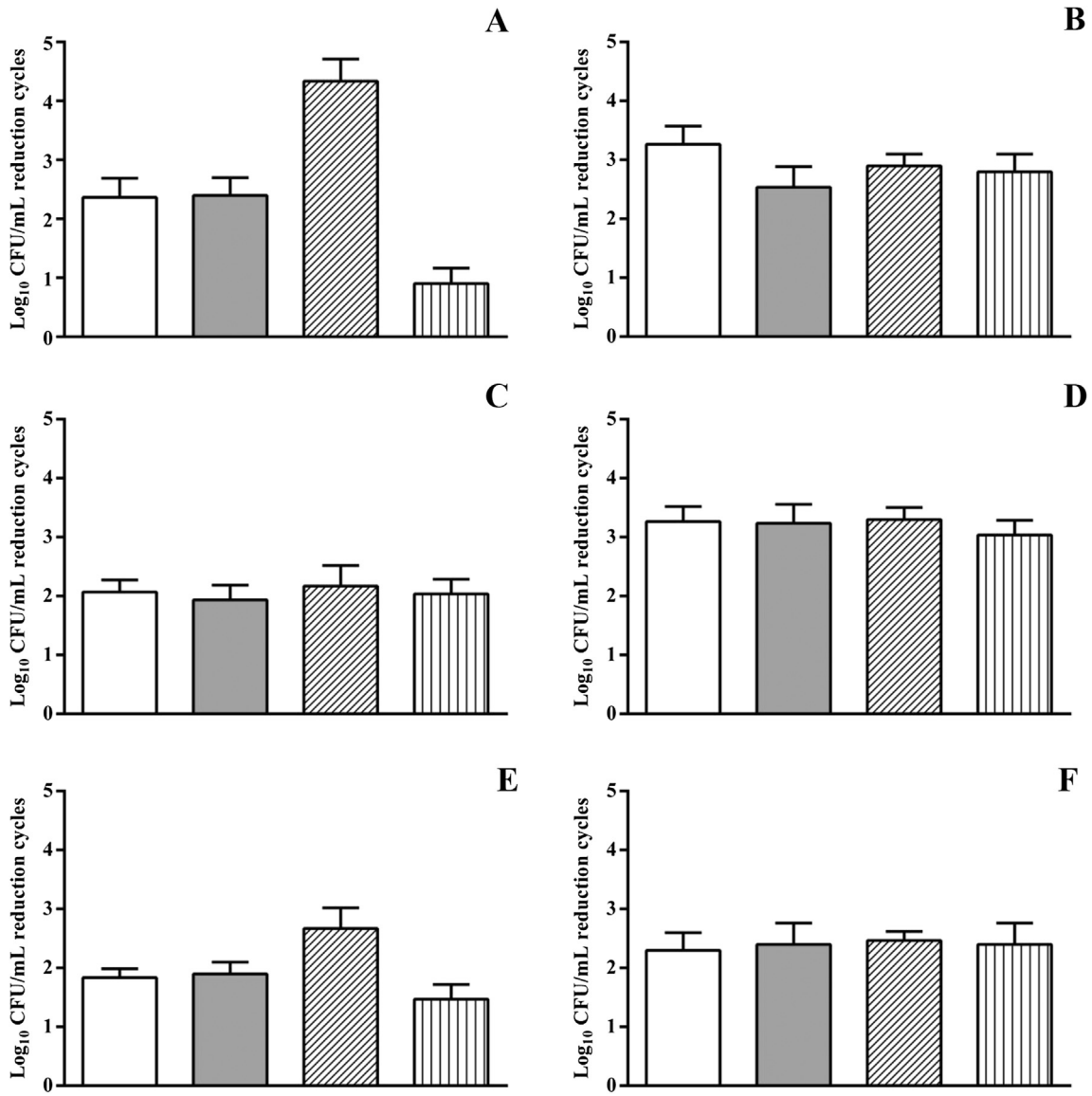

Fig. 3. Reduction cycles (Log10 CFU/mL) of the viable cell counts of aerobic mesophilic bacteria (A–B), fungi (C–D) and enterobacteria (E–F) in fresh leafy vegetables (28 °C) as a function of different concentrations of the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO) alone or in combination, after a 5-min (A, C, E) and 10-min (B, D, F) treatment (white bars: reduction caused by OVEO at its MIC: 0.6 μL/mL; grey bars: reduction caused by ROEO at its MIC: 10 μL/mL; diagonal dashed bars: reduction caused by 1/2 MIC OVEO: 0.3 μL/mL + 1/2 MIC ROEO: 5 μL/mL; vertical dashed bars: reduction caused by at 1/2 MIC OVEO: 0.3 μL/mL + 1/4 ROEO: 2.5 μL/mL.

Martos, 2013). This finding reinforced the consistency of the low MIC values found for OVEO, as thymol (phenolic) was found to be its major constituent, whereas eucalyptol (1,8 – cineole) (terpene hydrocarbon) was found to be the major constituent of ROEO. On the basis of the detected major constituents, the tested OVEO and ROEO were characterized as belonging to the thymol- and 1,8-cineol-chemotype, respectively (Jordan, Lax, Rota, Lorán, & Sotomayor, 2013; Russo, Galletti, Bocchini, & Carnacini, 1998). Generally, chemotype is a different chemical type of the same botanical species with a specific and genetically codified enzymatic equipment that directs its biosynthesis to the preferential formation of a definite compound (De Martino, Formisano, Mignola, & Senatore, 2009).

Although ROEO has often been found to have high MIC values against food-related bacteria, this EO is still considered a potential substance for antimicrobial use in foods, particularly vegetable products, given its beneficial impact on sensory aspects, primarily when applied in combination with EOs that could adversely affect the sensory acceptance of foods (Azerêdo et al., 2011; de Sousa et al., 2013). Though OVEO (which often has high amounts of thymol and/or carvacrol) has been reported to produce a changeable "warmly pungent flavor" in food during storage, ROEO (which often contains high amounts of eucalyptol) produces a changeable "distinctive but pleasant mint-like flavor" in the amounts that are usually proposed for use in foods. The combined application of OVEO and ROEO in vegetables could exploit a possible synergistic interaction and produce desirable antibacterial effects without causing noticeable undesirable changes to the food flavor and/or aroma. Consistent with this rationale, a previous study noted that the combination of OVEO and ROEO at subinhibtiory concentrations (1/4 MIC: 0.6 μL/mL and 1/4 MIC: 5 μL/mL, respectively) did not negatively impact the sensory attributes (appearance, texture, taste, odor, general perception and overall browning) and purchase intention of mix of fresh leafy vegetables (iceberg lettuce – *L. sativa*

L., beet – *B. vulgaris* L. var. cicla and rocket – *Eruca sativa* L.) over 72 h of refrigerated storage.

The FICI for the combined application of OVEO and ROEO was 0.5 against the mixed inoculum of *L. monocytogenes*, *E. coli* and *S.* Enteritidis, thus demonstrating a synergic interaction of these EOs. A synergistic interaction occurs when the combination of antimicrobials at subinhibitory concentrations (≤1/4 MIC) produces a greater inhibitory effect against the target organisms when compared to the inhibition caused by these antimicrobials when tested individually or at higher concentrations (Oliveira, Stamford, Gomes Neto, & de Souza, 2010). Regarding the general interpretation of the FICI assays, synergy could occur at a FICI ≤1 (combination of ≤1/2 MIC); however, because the dilution method can present a ±1 error, the dilution convention suggests that synergy is ≤ 0.5. However, a minor synergy (i.e., up to an FICI 1) may still be of practical importance (Gould et al., 1991). This was considered when choosing the subinhibitory concentrations of OVEO and ROEO (1/2 MIC and 1/4 MIC of each EO in different combinations) to use in assays of bacterial survival over time.

A previous study noted the same FICI (0.5) for the combined application of OVEO and ROEO against *Aeromonas hydrophila*, *L. monocytogenes* and *Pseudomonas fluorescens* in single populations (Azerêdo et al., 2011), whereas the combination of carvacrol and 1,8-cineol showed a FICI of 0.25 against the same bacteria in a mixed population (Oliveira et al., 2015). The authors stated that the enhanced inhibitory effects observed for OVEO and ROEO in combination could be partially explained by differences in the chemical structures of their major constituents (carvacrol/thymol and eucalyptol, respectively). These differences were predicted to result in distinct interactions with the target cell structures, which are primarily related to the antimicrobial activity of the tested EOs (Azerêdo et al., 2012; Sousa, Oliveira, Figueiredo, & Souza, 2015).

Ultrastructural studies of bacterial cells treated with carvacrol and eucalyptol have suggested that the oxygenated groups present in eucalyptol disturb the bacterial membrane structures, even when eucalyptol is present in the growth media at subinhibitory concentrations; therefore, eucalyptol could allow carvacrol to be more easily transported into the bacterial cells, where it can interact with different intracellular targets (de Sousa et al., 2013; Sousa et al., 2015). Overall, the presence of eucalyptol in the growth media was found to decrease the required amount of carvacrol needed to promote antimicrobial effects. Carvacrol is a thymol-isomer presenting structural differences due to the position of its hydroxyl group (OH group); however, carvacrol and thymol have similar mechanisms of action against bacterial cells (Lambert, Skandamis, Coote, & Nychas, 2001).

The FICI assay was used in combination with the time-kill studies given the availability of information about the kinetic of microbial inactivation, resulting in a dynamic picture of the antimicrobial interactions (Mackay, Milne, & Gould, 2000). The time-kill curves of bacteria exposed to OVEO and ROEO alone or in combination at the selected subinhibitory concentrations in vegetable broth revealed a significant decrease (>99% to ≥ 99.999%) in the viable counts over time, with a distinct behavior of *S.* Enteritidis challenged with ROEO at MIC. However, for most of the interactions, the highest reductions (>3 log cycles; 99.99%) were found in OVEO and ROEO at MIC and at the combinations 1/2 MIC OVEO + 1/2 MIC ROEO and 1/2 MIC OVEO + 1/4 MIC ROEO.

The OVEO and ROEO, alone or in combination at the selected subinhibitory concentrations, effectively decreased the counts of *L. monocytogenes*, *E. coli* and *S.* Enteritidis in artificially infected fresh leafy vegetables, as well as the counts of spoilage native flora (mesophilic bacteria, enterobacteria and fungi), after a 5- or 10-min treatment, although the effects were more pronounced as the exposure time increased. The inhibitory effects of the EOs alone or in combination against *L. monocytogenes*, *E. coli* and *S.* Enteritidis were lower in the fresh leafy vegetables than in the vegetable broth. This lower level of bacterial inactivation observed on the leafy vegetables following treatment with the EOs could be because the bacteria attach or infiltrate into the protective structures of the vegetables (lenticels, cuticle cells, broken trichomes and bruises), thereby impairing the contact with the individual constituents of the EOs (Azêredo et al., 2011; Burnett & Beuchat, 2001). Overall, the type of chemical agent, contact time, temperature, microbial load and chemical and physical properties of the vegetable surface could all have an influence on the bacterial response to sanitizers (Behrsing et al., 2000; Delaquis et al., 2002; Lee & Baek, 2008).

Given the data of bacterial survival obtained in this study, the following ranking of bacterial tolerance to the EOs tested was *S.* Enteritidis > *E. coli* > *L. monocytogenes*. This is not surprising because Gram-negative bacteria are generally less sensitive to EOs than Gram-positive bacteria (Hyldgaard, Mygind, & Meyer, 2012; Mazzarrino et al., 2015). In Gram-negative bacteria (such as *S.* Enteritidis and *E. coli*), this greater tolerance is due to the presence of the outer membrane, which limits the diffusion of hydrophobic compounds (such as the individual constituents of EOs) through lipopolysaccharide coverage (Vaara, 1992). Thus, the EO concentrations must be sufficient to allow diffusion through the external membrane and the lipid bilayer to reach the bacterial membrane for antibacterial effects to be established (de Oliveira, de Araújo Soares & Piccoli et al., 2013).

It is noteworthy that to successfully demonstrate sanitizing efficacy of any product intended for the treatment of food-contact surfaces, the product must demonstrate a 99.999% reduction (5 log cycles reduction) in the pathogen viable counts in suspension following a 30-s exposure to a pre-selected concentration (AOAC, 2013). However, to the best of our knowledge, no criteria regarding the inactivation level are currently available to substantiate the efficacy of sanitizers in vegetables and fruits. The treatment of raw vegetables in water containing chlorine (traditionally used as a sanitizer of fruits and vegetables) at concentrations of 50–250 ppm and with exposure times of 1–10 min can effectively decrease the population of pathogenic bacteria (including *L. monocytogenes*, *E. coli* and *Salmonella* spp.), aerobic microorganisms, psychrotrophic microorganisms, yeast and molds by 90–99.9% (1–3 log cycles) (FAO/WHO, 2008). Based on the available studies, the combinations of OVEO and ROEO at the selected subinhibitory concentrations were predicted to sanitize fresh vegetables infected with *L. monocytogenes*, *E. coli* and *S.* Enteritidis because they could reduce the initial population by 99–99.99% (2–3 log cycles) after 10 min of exposure. These results are also promising because products capable of establishing a ≥99.99% killing effect (≥3 log cycles reductions) are considered to possess bactericidal effects (LaPlante, 2007).

5. Conclusions

The results obtained revealed a synergistic interaction between OVEO and ROEO, as the combination of these EOs at subinhibitory concentrations (1/2 MIC OVEO + 1/2 ROEO and 1/2 MIC OVEO + 1/4 ROEO) against the pathogens associated with fresh leafy vegetable *L. monocytogenes*, *E. coli* and *S.* Enteritidis. The essential oils combined at subinhibitory concentrations were effective in decreasing the counts of these pathogenic bacteria in vegetable broth and in fresh leafy vegetables, as of the spoilage native flora in fresh leafy vegetables. Overall, these findings support the use of OVEO and ROEO at subinhibitory concentrations as alternatives to guarantee the safety and extend the shelf-life of fresh leafy vegetables.

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