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Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables

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ABSTRACT

This study assessed the occurrence of an enhancing inhibitory effect of the combined application of *Origanum vulgare* (OV) and *Rosmarinus officinalis* (RO) essential oils against bacteria associated to minimally processed vegetables using the determination of Fractional Inhibitory Concentration (FIC) index, kill-time assay in vegetal broth and application in vegetable matrices. Moreover, it was determined chemical composition of the essential oils and their effects alone and in mixture on sensory characteristics of minimally processed vegetables. Carvacrol (66.9 g/100 g) was the most prevalent compound in OV essential oil, while for RO was 1.8-cineole (32.2 g/100 g). OV and RO essential oil showed MIC in a range of 1.25–5 and 20–40 μL/mL, respectively. FIC indices of the combined application of the essential oils were 0.5 against *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophilla* suggesting a synergic interaction. Only for *Pseudomonas fluorescens* FIC index was 0.75 purposing additive effect. Application of the essential oils alone (MIC) or in mixture (¼ MIC + ¼ MIC or ¼ MIC + ½ MIC) in vegetable broth caused significant decrease ($p<0.05$) in bacterial count over 24 h. Mixture of essential oils reduced ($p<0.05$) the inocula of all bacteria in vegetable broth and in experimentally inoculated fresh-cut vegetables. Similar efficacy was found to reduce the autochthonous microflora in vegetables. Sensory evaluation of vegetables sanitized with essential oils revealed that the scores of the most evaluated attributes fell between like slightly and neither like nor dislike. The combination of essential oils at sub-inhibitory concentrations could mean an interesting approach to sanitize minimally processed vegetables.

1. Introduction

Minimally processed foods (MPF) are fresh, raw vegetables and fruits processed in order to supply a read-to-use product. The process of these products usually involves trimming, peeling, or cutting if necessary, washing and disinfection, followed for packaging in sealed pouches or on plastic trays sealed with polymeric films (Nguyen-the & Carlin, 1994). Regarding the current demand of consumers to convenient a ready-to-use foods possessing a fresh-like quality, the production of MPF has largely increased worldwide (Ragaert, Verbeke, Devlieghere, & Debevere, 2004).

The emergence of pathogenic microorganisms not previously associated with either raw or processed products has enhanced the potential for foodborne illness outbreaks associated with minimally processed products, since new processing and preservation techniques are able to create new ecology routes to microbial growth (Martin-Belloso, 2007). Fresh vegetables are susceptible to microbial attack after harvest due to the loss of natural resistance and their high water and nutrient content (Gutierrez, Bourke, Lonchamp, & Barry-Ryan, 2009). A wide range of fresh vegetables and unpasteurized juices has been implicated in outbreaks of foodborne diseases (Abadias, Usall, Anguera, Solsona, & Viñas, 2008).

Psychrotrophic pathogens (such as *Aeromonas hydrophilla*, *Listeria monocytogenes* and *Yersinia enterocolitica*) and spoilage bacteria (such as *Pseudomonas fluorescens* and *Enterobacteriaceae*) are cited to be the major microbiological concerns in minimally processed vegetables (Beuchat, 2002).

Disinfection using hypochlorite is often applied for MPF to improve safety and shelf-life, but this has presented some limitations and disadvantages, such as reduced antimicrobial efficacy and formation of carcinogenic chlorinated compounds (Gutierrez et al.,

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2009). The inefficacy of hypochlorite in eliminating bacteria in vegetables could be due to different factors: an aqueous hypochlorite solution may not wet the hydrophobic surface of the waxy cuticle of vegetables; the setting of a biofilm could protect the microorganisms against the lethal effects of the hypochlorite; and the contact with host tissue may inactivate hypochlorite (Carrasco, Pérez-Rodríguez, Valero, García-Gimeno, & Zurera, 2008). Contamination by pathogenic bacteria, their survival to disinfection processes and multiplication over refrigerated storage are serious concerns regarding MPF, since these products are consumed without major processing.

Currently there is an increasing consumer pressure for replacing chemically synthesized antimicrobial by natural alternatives in order to ensure food safety (Xu et al., 2007). The development and application of more natural sanitizers with a broad spectrum antimicrobial activity and no toxicity for human in MPF providing enhanced sensory quality and extended shelf-life is of interest to catering industry and consumers (Molinos et al., 2009). This trend prompts a particular increased interest in the use of essential oils as antimicrobial compounds to be applied in MPF.

Early studies showed that *Origanum vulgare* L. (oregano) and *Rosmarinus officinalis* L. (rosemary) essential oils possess interesting antimicrobial activity against spoilage and pathogenic food-related microorganisms (Oliveira, Stamford, Gomes Neto, & Souza, 2010; Souza, Barros, Conceição, Gomes Neto, & Costa, 2009). Early *in vitro* assays with essential oils showed they had promising antimicrobial properties, however when applied to food matrices amounts required to substantially inhibit the bacterial growth were often higher than would be organoleptically acceptable (Naveena, Muthukumar, Sen, Babji, & Murthy, 2006). Regarding that these high concentrations is likely to impart a certain flavor to foods, the addition of sub-inhibitory amounts of essential oils in mixtures may be a way to provide the balance between sensory acceptability and antimicrobial efficacy.

Here we report the investigation of an enhancing antibacterial effect when the essential oils from *O. vulgare* and *R. officinalis* were used in combination against autochthonous microflora and some bacteria associated with the contamination of minimally processed vegetables in food-based broth and food matrices. Moreover, the influence of these essential oils on the sensory attributes of minimally processed vegetables during refrigerated storage was investigated.

2. Material and methods

2.1. Essential oils

The essential oils from *O. vulgare* L. (batch OREORG01; density at 20 °C: 0.90; refractive index at 20 °C: 1.47) and *R. officinalis* L. (batch ROSTUN04; density at 20 °C: 0.94; refractive index at 20 °C: 1.51) were obtained from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brasil) and its quality parameters were described in an accompanying technical report. This supplier extracts essential oils on an industrial scale by steam distillation. The essential oils were assayed at concentrations ranging from 80 to 0.003 μL/mL. Essential oils solutions were prepared in nutrient broth (Himedia, India) using bacteriological agar (1.5 g/L) as stabilizing agent (Souza et al., 2009).

2.2. Bacterial strains

L. monocytogenes ATCC 7644, *Y. enterocolitica* ATCC 9610, *A. hydrophilla* INCQS 7966 and *P. fluorescens* ATCC 11253 obtained from the Microorganism Collection, Laboratory of Food Microbiology, Federal University of Paraíba, João Pessoa, Brazil were used as test microorganisms. Stock cultures were kept on nutrient agar (Himedia, India) slants under refrigeration (6 °C).

Inocula used in antimicrobial assays were obtained from overnight cultures grown on nutrient agar slants at 35 °C for *L. monocytogenes* and *Y. enterocolitica*, and at 28 °C for *A. hydrophilla* and *P. fluorescens*. A loopfull of the culture was diluted in sterile saline solution (8.5 g/L) to have a final concentration of approximately 10^8 colony forming unit per mL (cfu/mL) adjusted according to the turbidity of 0.5 McFarland standard tube.

2.3. Preparation of vegetable broth

Iceberg lettuce (*Lactuca sativa* L.), beet (*Beta vulgaris* L. var. cicla, L.) and rocket (*Eruca sativa* L.) were purchased from a local wholesale market in João Pessoa (Brazil) on the day of harvest and transported within 20 min under refrigerated conditions. A mixture (1:1:1) of the samples containing 200 g of the vegetal material was mashed with 800 mL of distilled water using a domestic blender and vacuum filtered using Whatman no. 1 filter paper. The obtained material was sterilized by filtration using a Millipore 0.22 μm.

2.4. Essential oil chemical analysis

The chemical composition of the essential oils was analyzed using a gas chromatograph (GC) fitted to a mass spectrometer (MS) (Shimadzu GC/MS-QP5050A) operating in electron-impact (70 eV, *m/z* 50–400) mode; fused silica capillary column used was 5% phenyl dimethylpolysiloxane (DB-5MS, J & W Scientific, Folsom, CA) with length 30 m, 0.25 mm i.d., 0.1 μm film thickness. The analyze of the oil was performed employing the following conditions: sample preparation 1 μL in 1 mL of hexane; injection volume 1 μL; split ratio 1:5; helium flow rate 1.6 mL/min; temperature programme ramp from 60 °C to 200 °C with a gradient of 5 °C/min (holding the initial and final temperature for 5 min); injector temperature 260 °C; detector temperature 280 °C; mass spectra: electron impact 70 eV; mass scanning rate: 40–550 amu; scan time: 0.5 s.

The identification of the essential oil components was performed by retention indexes and comparing their mass spectra with a data bank (System GC-MS, Nist. 62 lib) and literature (Adams, 1995). Retention indexes were obtained by co-injection with a hydrocarbons (C_9–C_{24}) standard mixture using the van Den Dool & Kratz equation (van Den Dool & Kratz, 1963).

2.5. Determination of the minimum inhibitory concentration (mic) and minimum bactericidal concentration (mbc)

MIC values of the essential oils were determined using the macrodilution in broth procedure. Four milliliters of double strength nutrient broth (Himeida, India) was inoculated with 1 mL of the bacterial inocula, mixed with 5 mL of two fold dilutions of the essential oil at concentrations ranging of 160 to 0.06 μL/mL, and followed by shaking for 30 s using vortex. The system was statically incubated for 24 h at 35 °C for *L. monocytogenes* and *Y. enterocolitica*, and at 28 °C for *A. hydrophilla* and *P. fluorescens*. MIC was defined as the lowest concentration of the essential oils required for preventing visible bacterial growth. Control flasks without the essential oils were tested similarly (Nostro, Cannatelli, Musolino, Procopio, & Alonzo, 2002).

2.6. Synergy assays

The assays of synergy of the essential oils were carried out by determining Fractional Inhibitory Concentration (FIC) index in nutrient broth using the macrodilution method. FIC was calculated as follows: MIC of the combination of the essential oils/MIC of the essential oil alone. Essential oils were combined at MIC + MIC; MIC + ½ MIC; MIC + ¼ MIC; ½ MIC + ½ MIC; ¼ MIC + ¼ MIC; and ½ MIC + ¼ MIC. Synergy was FIC ≤ 0.5; addition was FIC > 0.5 to 4; and antagonism was FIC > 4 (Mackay, Milne, & Gould, 2000; Oliveira et al., 2010).

2.7. Time-kill assay

The effect of the essential oils alone (MIC) and in mixture (¼ MIC + ¼ MIC or ¼ MIC + ½ MIC) on the cell viability of the bacterial strains in vegetable broth over 96 h was evaluated by the viable cell count procedure. For this, 4 mL of vegetable broth was inoculated with 1 mL of the bacterial inocula and 5 mL of the essential oils solutions alone (MIC) or in mixture (¼ MIC + ¼ MIC or ¼ MIC + ½ MIC) were added to the system and gently shaken for 30 s. The system was incubated at 37 °C for *L. monocytogenes* and *Y. enterocolitica*, and at 28 [°C for *A. hydrophilla* and *P. fluorescens*. At different time intervals (0, 2, 4, 8 12 and 24 h), 1 mL of the suspension was serially diluted (10^{-1}–10^{-5}) in sterile peptone water (1 g/L) and inoculated on nutrient agar Petri dishes for 24 h at 35 °C or 28 °C (Barros et al., 2009). Control flasks without essential oils were tested similarly. The results were expressed in log of cfu/mL.

2.8. Effect of essential oils on survival of bacteria in fresh vegetable

Portions of 90 g of a pool of iceberg lettuce, beet and rocket (in a rate of 1:1:1) previously washed with sterile distilled water were shredded by glove-covered hands and inoculated with the bacteria according to the following procedure: the portion of vegetables was submerged in 900 mL of the bacterial inoculum (*L. monocytogenes, Y. enterocolitica, A. hydrophilla* and *P. fluorescens*, approximately 10^8 cfu/mL), softly rotated with a sterile glass stem for 5 min to ensure even inoculation, and air-dried for 1 h in a bio-safety cabinet. After that, the vegetables were submerged in 250 mL of the solutions of essential oils alone (MIC) or in mixture (¼ MIC + ¼ MIC or ¼ MIC + ½ MIC) for 5 min at 28 °C. Then, a 25 g sample of the vegetables was aseptically taken and transferred into a sterile stomacher bag containing 225 mL of sterile peptone water (1 g/L) and homogenized for 60 s. Subsequently, a decimal dilution series (10^{-2}–10^{-5}) was made in the same diluent and bacteria enumeration was performed by pour-plating 0.1 mL of the appropriate sample dilution on *Listeria* Selective Agar Base + *Listeria* Selective Supplement II (Himedia, India) at 37 °C (24 h) for *L. monocytogenes* count; *Yersinia* Selective Agar Base + *Yersinia* selective supplement (Himedia, India) at 37 °C (24 h) for *Y. enterocolitica* count; *Aeromonas* isolation Medium + *Aeromonas* Selective Supplement (Himedia, India) at 28 °C (48 h) for *A. hydrophilla* count; and *Pseudomonas* Agar Base + CFC Supplement (Himedia, India) at 28 °C (48 h) for *P. fluorescens* count (Xu et al., 2007). Control flasks containing sterile distilled water were tested in the same way. The results were expressed in log of cfu/mL.

2.9. Effect of essential oils on survival of natural flora in fresh vegetable

Portions of 90 g of the lettuce, beet and rocket (in a rate of 1:1:1) were shredded by glove-covered hands and immediately submerged in 250 mL of the essential oils solutions alone (MIC) or in mixture (¼ MIC + ¼ MIC or ¼ MIC + ½ MIC), and softly rotated for 5 min at 28 °C using a sterile glass stem for ensure complete coverage and contact of surfaces with the essential oil solutions. Then, a 25 g sample of the vegetables was aseptically taken and transferred into a sterile stomacher bag containing 225 mL of sterile peptone water (1 g/L) and homogenized for 60 s. Subsequently, a decimal dilution series (10^{-2}–10^{-5}) was made in the same diluent and enumeration of the natural 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 Sabouraud agar (Himedia, India) at 28 °C (48–72 h) for fungi. The results were expressed in log of cfu/mL (Lopéz-Galvéz et al., 2010). Control flasks containing sterile distilled water were tested in the same way.

2.10. Sensory evaluation

Sensory evaluation was performed by the acceptance test using 50 experienced members pre-selected according to interest and fresh leafy consuming habits. Panelists worked in individual booths with controlled conditions of temperature and lighting. Portions of 180 g of a pool of iceberg lettuce, beet and rocket (in a rate of 1:1:1) previously washed with sterile distilled water were shredded by glove-covered hands, submerged in 500 mL of the essential oils solutions alone (MIC) or in mixture (¼ MIC + ¼ MIC), softly rotated for 5 min at 28 °C using a sterile glass stem for 5 min, air-dried for 30 min in a bio-safety cabinet and put on plastic trays sealed with polypropylene film. After 24, 48 and 72 h of storage at 7 °C, the panelists were served with 20 g of each sample coded with three-digit random numbers placed on small white plates and served immediately after being taken out of the refrigerated storage. Panelists were asked to use low-salt crackers and water to clean their palates between the assessed samples. The acceptance of appearance, texture, taste, odor and general perception were evaluated on a 5-point hedonic scale, ranging from 1 (dislike very much) to 5 (like very much). Still, the panelists were asked to assess the edge vascular tissue browning and overall browning of the vegetables. The purchasing intention was evaluated using a 5-point hedonic scale, ranging from 1 (certainly would not purchase) to 5 (certainly would purchase). Samples of vegetables without exposure to essential oils were tested similarly as control.

2.11. Reproducibility and statistics

All assays were made in triplicate on three separate occasions, and the results were expressed as average of the assays. Statistical analysis was performed to determine significant differences ($p<0.05$) by ANOVA followed by Duncan test. For this, Sigma stat 3.1 computer program was used.

3. Results

3.1. Essential oil chemical analysis

As shown in Tables 1 and 2, GC-MS analysis resulted in the identification of 16 and 13 compounds in amounts higher than 0.1 g/100 g of the total mass in the essential oil of *O. vulgare* and *R. officinalis*, respectively. Carvacrol (66.9 g/100 g) was the most prevalent compound in *O. vulgare* essential oil, followed for *p*-cymene (13.9 g/100 g) and γ-terpinene (7.8 g/100 g). Other compounds as myrcene (1.9 g/100 g), α-pinene (1.8 g/100 g), α-terpinene (1.6 g/100 g), β-caryophyllene (1.6 g/100 g) and linalool (1.1 g/100 g) were found in minor amounts.

Table 1
GC-MS analysis of the essential oil from *Origanum vulgare* L. leaves.[a]

Peaks	Retention index	Compound	Amount in the oil (g/100 g)
1	2489	α-Thujene	0.4
2	2566	α-Pinene	1.8
3	2768	Camphene	0.7
4	3254	β-Pinene	0.6
5	3538	Myrcene	1.9
6	3973	δ-3-Careen	0.1
7	4718	α-Terpinene	1.6
8	4219	*p*-Cymene	13.9
9	4712	Limonene	0.3
10	4818	1,8-Cineole	0.6
11	5195	*cis*-Ocimene	0.2
12	5458	*trans*-Ocimene	0.3
13	5919	γ-Terpinene	7.8
14	6996	Linalool	1.1
15	8273	Carvacrol	66.9
16	8478	β-Cariophyllene	1.6

[a] Amounts lower than 0.1 g/100 g were excluded.

Table 2
GC-MS analysis of the essential oil from *Rosmarinus officinalis* L. leaves.[a]

Peaks	Retention index	Compound	Amount in the oil (g/100 g)
2	2445	α-Pinene	14.2
3	2668	Camphene	8.2
4	3415	β-Pinene	7.0
6	3724	Myrcene	1.8
9	4489	Limonene	5.6
10	4574	1,8-Cineole	32.2
12	4732	α-Terpinene	1.1
14	4812	*p*-Cymene	3.3
17	5356	Camphor	15.2
19	7138	Bornyl-acetate	1.1
20	8478	β-Cariophyllene	2.3
25	7998	Verbenone	2.3
26	8671	Borneol	1.9

[a] Amounts lower than 0.1 g/100 g were excluded.

For the essential oil from *R. officinalis* the compounds found in higher amounts were 1,8-cineole (32.2 g/100 g), camphor (15.2 g/100 g), α-pinene (14.2 g/100 g), camphene (8.2 g/100 g), β-pinene (7 g/100 g) and limonene (5.6 g/100 g). The other indentified compounds were in a range of 1.1–3.3 g/100 g.

3.2. MIC of the essential oils

Results of the MIC values of *O. vulgare* and *R. officinalis* essential oils against bacteria associated to minimally processed vegetables are shown in Table 3. *O. vulgare* and *R. officinalis* essential oil showed MIC in a range of 1.25–5 and 20–40 μL/mL, respectively. Highest MIC values for both oils were found against *P. fluorescens*. MIC values of *R. officinalis* were 8 to 16 fold-higher than those found for *O. vulgare*.

3.3. FIC index of the combined application of essential oils

FIC indexes for the combined application of *O. vulgare* and *R. officinalis* essential oils were 0.5 for *L. monocytogenes*, *Y. enterocolitica* and *A. hydrophilla* suggesting a synergic interaction of the essential oils against these bacteria. The oils inhibited the growth of these bacteria when applied in combination of ¼ MIC + ¼ MIC. Only for *P. fluorescens*, the FIC index was 0.75 revealing an additive effect. Inhibition of *P. fluorescens* was noted when the essential oils from *O. vulgare* and *R. officinalis* were combined at ¼ MIC + ½ MIC, respectively.

Test strains presented capability to grow at sub-inhibitory concentrations (½ MIC and ¼ MIC) of both oils when applied alone.

3.4. Kill-time assays

Kill-time of bacteria associated to minimally processed vegetables when exposed to *O. vulgare* and *R. officinalis* essential oils alone and in combination in vegetable broth over 24 h is given in Figs. 1 to 4. The oils were assayed at their MIC and at ¼ MIC in combination, excepting against *P. fluorescens* where the essential oil of *R. officinalis vulgare* was tested at ½ MIC regarding the obtained results of the FIC index.

Table 3
Minimum inhibitory concentrations of the essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. against bacteria associated to minimally processed vegetables.

Bacterial strains	Essential oil (μL/mL)	
	O. vulgare	*R. officinalis*
L. monocytogenes ATCC 7644	1.25	20
Y. enterocolitica ATCC 9610	2.5	20
A. hydrophilla INCQS 7966	2.5	20
P. fluorescens ATCC 11253	5	40

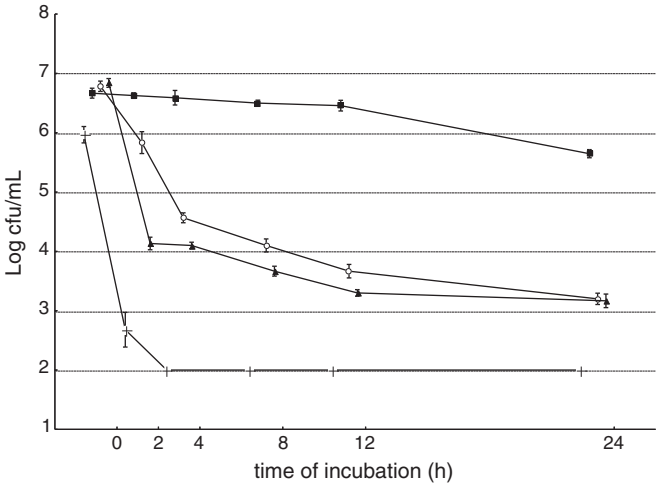

Fig. 1. Survivors curves for *L. monocytogenes* ATCC 7644 in vegetable broth at 37 °C as a function of antimicrobial concentration: (■): control (0 μL/mL); (+): *O. vulgare* essential oil (MIC: 1.25 μL/mL); (○): *R. officinalis* essential oil (MIC: 20 μL/mL; (▲): *O. vulgare* essential oil (¼ MIC: 0.31 μL/mL) + *R. officinalis* essential oil (¼ MIC: 5 μL/mL).

Addition of the essential oils from *O. vulgare* and *R. officinalis* at their MIC values resulted in significant drop ($p<0.05$) in the bacterial counts over 24 h. Values lower than two log cycles were found for *Y. enterocolitica*, *A. hydrophilla* and *P. fluorescens* in broth added of the essential oils alone already after 4 h, and these counts were maintained in the remainder evaluated times. Smaller decrease in viability of *L. monocytogenes* was found in the broth added of the essential oil from *R. officinalis* when counts in a range of 3–4 log cycles were noted from 4 h onward.

The combination of essential oils at sub-inhibitory concentrations reduced the initial inocula of *Y. enterocolitica*, *A. hydrophilla* and *P. fluorescens* to 2 log cycles after a maximum time of 4 h and no recovery in viable count was noted in the remainder evaluated intervals. For *L. monocytogenes*, although the mixture of essential oils had caused a linear and significant reduction ($p<0.05$) over time, these counts were always higher than 3 log cycles. No significant difference ($p>0.05$) was found among the counts of *Y. enterocolitica*, *A. hydrophilla* and *P. fluorescens* for the broth added of the essential oils alone or in combination. The counts found for *L. monocytogenes* in the broth added of the essential oil from *O. vulgare* alone were

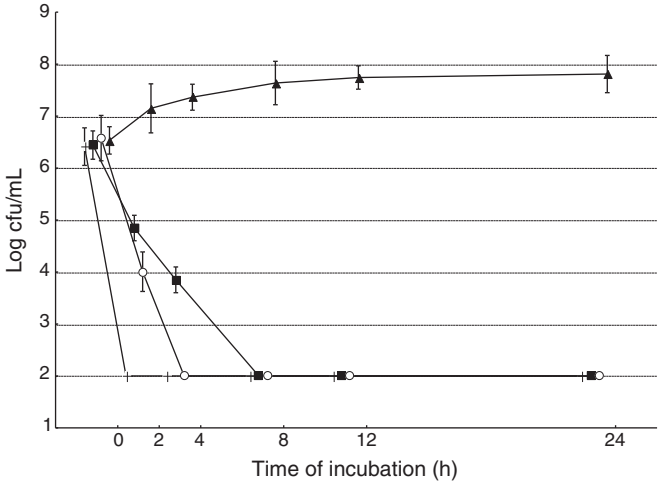

Fig. 2. Survivors curves for *Y. enterocolitica* ATCC 9610 in vegetable broth at 37 °C as a function of antimicrobial concentration: (■): control (0 μL/mL); (+): *O. vulgare* essential oil (MIC: 2.5 μL/mL); (○): *R. officinalis* essential oil (MIC: 20 μL/mL; (▲): *O. vulgare* essential oil (¼ MIC: 0.62 μL/mL) + *R. officinalis* essential oil (¼ MIC: 5 μL/mL).

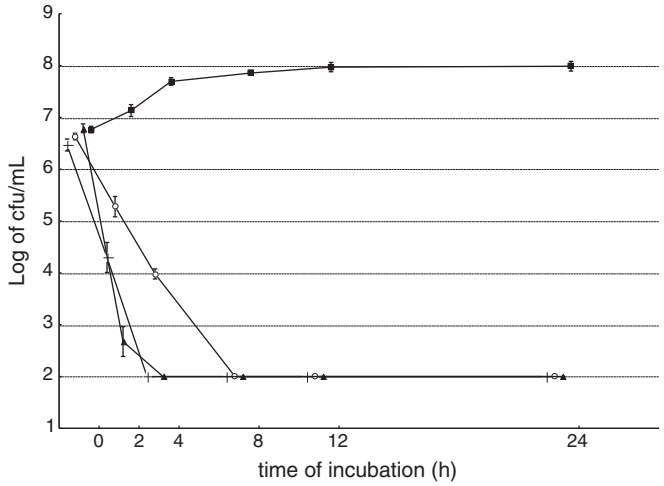

Fig. 3. Survivors curves for *A. hydrophilla* INCQS 7966 in vegetable broth at 28 °C as a function of antimicrobial concentration: (■): control (0 μL/mL); (+): *O. vulgare* essential oil (MIC: 2.5 μL/mL); (○): *R. officinalis* essential oil (MIC: 20 μL/mL; (▲): *O. vulgare* essential oil (¼ MIC: 0.62 μL/mL) + *R. officinalis* essential oil (¼ MIC: 5 μL/mL).

different ($p<0.05$) to those obtained for the broth added of the essential oil from *R. officinalis* alone and of both oils in mixture.

The application of the essential oils alone or in mixture caused significant decrease ($p<0.05$) in bacterial counts over the evaluated intervals in comparison to the control assay.

3.5. Effect of essential oils on survival of bacteria in hand-cut fresh vegetables

Effect of *O. vulgare* and *R. officinalis* essential oils alone and in mixture on the counts of *L. monocytogenes, Y. enterocolitica, A. hydrophilla* and *P. fluorescens* in experimentally inoculated hand-cut fresh vegetables are shown in Table 4. The application of the essential oils alone (MIC values) or in mixtures (sub-inhibitory concentrations) in vegetables caused significant reduction ($p<0.05$) in bacterial count in comparison to the control assay. The mixture of essential oils reduced the initial inocula of all tested bacteria from approximately 8 to 5 log cfu/g after 5 min of exposure. Exposure of vegetables to the MIC of *O. vulgare* essential oil (1.25–5 μL/mL) provided the more intense drop in the bacterial counts, which were between <1.0 and 2.7 log cfu/g.

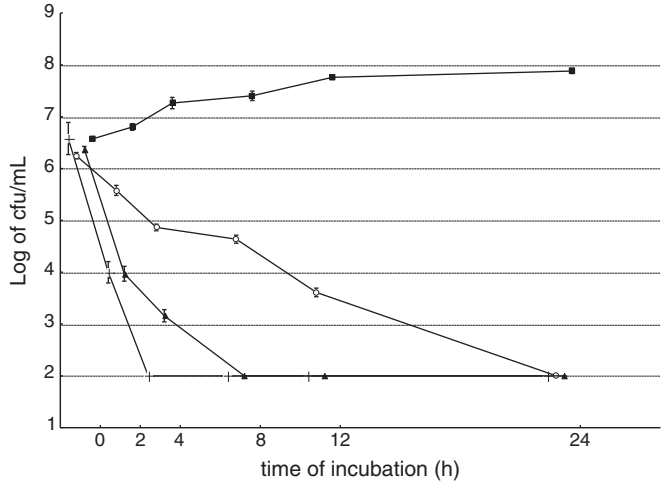

Fig. 4. Survivors curves for *P. fluorescens* ATCC 11253 in vegetable broth at 28 °C as a function of antimicrobial concentration: (■): control (0 μL/mL); (+): *O. vulgare* essential oil (MIC: 5 μL/mL); (○): *R. officinalis* essential oil (MIC: 40 μL/mL; (▲): *O. vulgare* essential oil (¼ MIC: 1.25 μL/mL) + *R. officinalis* essential oil (½ MIC: 10 μL/mL).

Bacterial counts found for the vegetables exposed to *O. vulgare* essential oil alone were significantly lower ($p<0.05$) than those obtained for the mixture of essential oils and for *R. officinalis* essential oil alone. No difference ($p>0.05$) was found for the counts in vegetables exposed to the mixture of essential oils and to *R. officinalis* essential oil alone.

3.6. Effect of essential oils on survival of autochthonous microflora in hand-cut fresh vegetables

Effect of *O. vulgare* and *R. officinalis* essential oils alone and in mixture on the counts of mesophilic bacteria, psychrotrophic bacteria, *Enterobacteriaceae* and fungi in hand-cut fresh vegetables are shown in Table 5. The application of essential oils alone (MIC values) or in mixtures (sub-inhibitory concentrations) caused significant reduction ($p<0.05$) of the microflora of fresh-cut vegetables. In accordance with the results found in experimentally inoculated vegetables, the application of *O. vulgare* essential oil alone caused the highest decrease in the counts of all assessed groups (or family) of microorganisms. No difference ($p>0.05$) was found for the microbial counts in vegetables exposed to the mixture of essential oils and to *R. officinalis* essential oil alone, except for mesophilic.

3.7. Sensory analysis

Results of the analysis for sensory scores in vegetables sanitized with *O. vulgare* and *R. officinalis* essential oils alone and in mixture are shown in Table 6. The mean of the most evaluated attributes fell between “like slightly” and “neither like nor dislike” on the hedonic scale for samples treated with essential oils alone and in mixture. At all assessed storage times no significant difference was found for odor and taste in samples sanitized with the mixture of essential oils (¼ MIC + ¼ MIC) and control. Still, no significant difference ($p>0.05$) among these samples was found for all tested attributes after 72 h of storage. In general, small scores ($p<0.05$) for vegetables sanitized with essential oils alone (MIC) were found after 72 h of storage. The time of storage showed no influence on the sensory attributes of vegetables sanitized with the mixture of essential oils, excepting to cut edge tissue browning.

When asked to report about the purchase intention panelists revealed no difference ($p>0.05$) only in respect of vegetables treated with the essential oils in mixture and the control after 48 and 72 h of storage attributing scores fell between “maybe would purchase/maybe would not purchase” and “possibly could purchase”.

4. Discussion

The efficacy of the combined use of the essential oils from *O. vulgare* and *R. officinalis* at sub-inhibitory concentrations in inhibiting the survival of some pathogenic and spoilage bacteria and autochthonous associated to minimally processed vegetables was assessed in this study. Although both tested essential oils have been effective in inhibiting all assayed bacterial strains, the MIC values found for the oils were widely different against the same test bacteria.

Lower values (8 to 16 fold) were ever noted to *O. vulgare* essential oil. These results suggest *O. vulgare* essential oil as possessing some compounds able to establish their antibacterial activity in lower amounts in comparison to the compounds found in *R. officinalis*. Smallest MIC values noted to *O. vulgare* could be associated to the phenolic carvacrol, the major component of this oil, which has been related to the highest antibacterial properties of essential oils (Barros et al., 2009; Nostro et al., 2002). The main compounds present in *R. officinalis* essential oil were 1,8-cineole, camphor and α-pinene. *P. fluorescens* presented as the most resistant strain to the tested

Table 4
Counts (log cfu/g) of *L. monocytogenes, Y. enterocolitica, A. hydrophilla* and *P. fluorescens* in experimentally inoculated fresh-cut vegetables exposed to the essential oils from *O. vulgare* and *R. officinalis* (alone and in mixture) for 5 min (28 °C).

Treatment	Bacterial strains			
	L. monocytogenes ATCC 7644	*Y. enterocolitica* ATCC 9610	*A. hydrophilla* INCQS 7966	*P. fluorescens* ATCC 11253
O. vulgare essential oil (MIC)	<1.0 ^{C}	2.4 (± 0.1) ^{C}	<1.0 ^{C}	2.7 (± 0.3) ^{C}
R. officinalis essential oil (MIC)	4.8 (± 0.3) ^{B}	5.7 (± 0.4) ^{B}	4.9 (± 0.2) ^{B}	5. 7 (± 0.4) ^{B}
Mixture of essential oils [a]	5.3 (± 0.2) ^{AB*}	5. 6 (± 0.3) ^{B}	5.4 (± 0.1) ^{B*}	5.3 (± 0.3) ^{B}
Control/water	7.5 (± 0.3) ^{A}	7.7 (± 0.3) ^{A}	7.9 (± 0.3) ^{A}	7.6 (± 0.2) ^{A}

Means in the same raw with different capital lowercase letters are significantly different ($p<0.05$) according to Duncan test.
[a] ¼ MIC of *O. vulgare* + ¼ MIC of *R. officinalis* for *L. monocytogenes*, *Y. enterocolitica* and *A. hydrophilla*; ¼ MIC of *O. vulgare* + ½ MIC of *R. officinalis* for *P. fluorescens.*

essential oils when regarded the highest MIC values in comparison to the other bacteria. The resistance of *Pseudomonas* spp. to some essential oils has been previously cited for other researchers (Burt, 2004; Holley & Patel, 2005).

The synergy of *O. vulgare* and *R. officinalis* essential oils against bacteria has not been reported before. From our results, FIC indices assays suggested a synergistic effect of the combined application of these oils against *L. monocytogenes*, *Y. enterocolitica* and *A. hydrophilla*. Combination of the essential oils was additive only against *P. fluorescens*, which were in accordance with the results of its highest resistance in the assays of MIC determination.

Time-kill curves of the essential oils alone and combined at selected sub-inhibitory amounts in vegetable broth also revealed an interesting inhibition of the viability of all tested strains over 24 h. For the most interactions, the combination of essential oils at sub-inhibitory concentrations caused an inhibition in cell viability similar to that found when the oils were tested individually at their MIC.

Anti-microbial effect of essential oils alone, and mainly when applied in mixtures of sub-inhibitory concentrations, was smaller in fresh-cut vegetables when compared to its addition in vegetable broth. Factors responsible for the smaller efficacy of essential oils as sanitizers in fresh-cut vegetables could be the high initial microbial load; the pre-harvest contamination by which the bacteria have possibility to attach or infiltrate into protective structures of vegetables (lenticels, broken trichomes and bruises) impairing the contact of essential oils with target bacteria; and the presence of other remainder organic material, such as soil particles structures (Burnett & Beuchat, 2001).

Few studies have used FIC calculations for assessing the occurrence of synergy resulting of the mixture of essential oils. Gutierrez, Barry-Ryan, and Bourke (2008) found additive effect of the combination of *O. vulgare* with *Thymus vulgaris* (thyme) essential oils against *B. cereus*, and indifference against *Escherichia coli*, *L. monocytogenes* and *Pseudomoans aeruginosa*. Gutierrez et al. (2009) found additive effect of the combination of these oils against *Enterobacter cloacae*, *P. fluorescens* and *Listeria innocua*. It seems reasonable that combination of essential oils possessing compounds with similar structures may exhibit additive rather than synergistic effect. The occurrence of additive interaction of these essential oils could be related to their similar composition possessing phenolics (carvacrol and thymol) as main compounds, suggesting a similar mechanism of action.

It has been hypothesized that these phenolic active compounds, such as carvacrol and thymol, sensitize the membrane, and when saturation of these sites occurs, there is a gross damage and sudden collapse of integrity of the bacterial cytoplasmic membrane and leakage of vital intracellular constituents (Rhayour, Bouchikhi, Tantaoui-Elaraki, Sendide, & Remmal, 2003). Increased concentrations of phenolic compounds in growth media have not represented a progressive increase in their antibacterial activity because of the limitation of their complexation to available targets (amino acids and proteins) in the cells (Juven, Kanner, Schved, & Weisslowicz, 1994).

Previous studies have reported enhanced antimicrobial effect of the combination of different concentrations of plant substances as essential oils, extracts or phytochemicals. Fu et al. (2007) noted increased antimicrobial effect caused by the combined use (1:5, 1:7 and 1:9) of the essential oils of *Syzigium aromaticum* (clove) and *R. officinalis* against *Candida albicans*. In other study, Lin, Labbe, and Shetty (2005) noted increased inhibitory effect toward *Vibrio parahaemolyticus* when aqueous extracts of oregano and cramberry were tested in a ratio of 1:1 in comparison to their application individually.

Regarding the results of percent of each compound in the essential oils and the MIC values it can be suggested an amount of carvacrol (66.9 g/100 g) and 1,8-cineole (32.2 g/100 g) in a range of 0.84–3.35 μL/mL and 12.9–6.45 μL/mL in the solutions of the essential oils of *O. vulgare* and *R. officinallis*, respectively, found as MIC. In the solutions used to the combination of ¼ MIC of *O. vulgare* and ¼ MIC of *R. officinallis* the amount of carvacrol and 1,8-cineole was approximately of 0.42 and 5 μL/mL, respectively, as applied against *Y. enterocolitica* and *A. hydrophilla,* while to *L. monocytogenes* it was 0.31 and 4 μL/mL. In the case of combination of ¼ MIC of *O. vulgare* and ½ MIC of *R. officinallis* as used against *P. fluorescens*, the amount of carvacrol and 1,8-cineole was approximately of 0.84 and 20 μL/mL, respectively.

It is difficult to understand the exact mechanism for the establishment of the synergistic effect caused by the combined application of the essential oils of *O. vulgare* and *R. officinalis*.

Table 5
Counts (log cfu/g) of the microflora of fresh-cut vegetables exposed to essential oils from *O. vulgare* and *R. officinalis* (alone and in mixture) for 5 min (28 °C).

Treatment	Microorganisms			
	Mesophilics	Psychrotrophic	Enterobaceriaceae	Moulds and yeasts
O. vulgare essential oil (MIC)	<1.0 ^{D}	<1.0 ^{C}	2.2(± 0.1) ^{C}	2.7(± 0.2) ^{C}
R. officinalis essential oil (MIC)	3.5 (± 0.3) ^{C}	3.3 (± 0.2)^{B}	4.5 (± 0.3) ^{B}	5.1 (± 0.3) ^{B}
Mixture of essential oils [a]	4.6 (± 0.2) ^{B}	4.1(± 0.1) ^{B}	4.7 (± 0.2) ^{B}	5.2 (± 0.2) ^{B}
Control (water)	7.3 (± 0.3) ^{A}	6.6 (± 0.4) ^{A}	7.1 (± 0.4) ^{A}	6.7 (± 0.4) ^{A}

Means in the same column with different superscript capital letters are significantly different ($p<0.05$) according to the Duncan test.
[a] ¼ MIC + ¼ MIC.

Table 6
Mean sensory scores for minimally processed leafy vegetables sanitized with essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. alone and in mixture over refrigerated storage.

Attributes	Time of storage	*O. vulgare* (MIC)	*R. officinalis* (MIC)	FIC index (¼ MIC + ¼ MIC)	Control
Apperance	24	3.46 ± 1.07^{Ab}	3.74 ± 0.92^{Aab}	3.64 ± 0.96^{Ab}	4.14 ± 0.85^{Aa}
	48	3.16 ± 1.11^{ABb}	3.34 ± 0.98^{Ab}	3.42 ± 0.90^{Ab}	4.54 ± 0.67^{Aa}
	72	2.9 ± 0.95^{Bb}	2.5 ± 0.93^{Bb}	3.44 ± 0.99^{Aa}	3.72 ± 1.03^{Ba}
Cut edge vascular tissue browning	24	3.58 ± 1.01^{Ab}	3.6 ± 1.03^{Ab}	3.64 ± 1.06^{Ab}	4.26 ± 0.94^{Aa}
	48	3.18 ± 1.04^{ABb}	3.5 ± 0.97^{Ab}	3.1 ± 0.88^{Bb}	4.7 ± 0.50^{Ba}
	72	2.7 ± 1.03^{Bb}	2.32 ± 0.86^{Bb}	3.5 ± 0.86^{ABa}	3.68 ± 0.89^{Ca}
Overall browning	24	3.66 ± 1.02^{Ab}	4.06 ± 0.86^{Aab}	3.74 ± 0.98^{Aab}	4.22 ± 0.93^{Aa}
	48	3.68 ± 0.91^{Ab}	3.78 ± 0.86^{Ab}	3.68 ± 0.81^{Ab}	4.56 ± 0.81^{Aa}
	72	2.98 ± 1.00^{Bb}	2.62 ± 0.98^{Bb}	3.70 ± 0.76^{Aa}	3.80 ± 0.88^{Ba}
Texture	24	3.90 ± 0.93^{Aa}	4.02 ± 1.04^{Aa}	4.12 ± 0.98^{Aa}	4.34 ± 0.74^{Aa}
	48	3.80 ± 1.08^{Ab}	3.84 ± 0.91^{ABb}	3.96 ± 0.94^{Ab}	4.74 ± 0.44^{Ba}
	72	3.88 ± 0.91^{Aab}	3.44 ± 0.95^{Bb}	4.04 ± 0.85^{Aa}	4.12 ± 0.84^{ACa}
Taste	24	3.62 ± 1.17^{Aa}	3.64 ± 1.30^{Aa}	3.76 ± 1.06^{Aa}	3.92 ± 0.94^{Aa}
	48	3.64 ± 0.94^{Ab}	3.54 ± 1.21^{Ab}	3.9 ± 1.03^{Aab}	4.4 ± 0.75^{Ba}
	72	3.68 ± 0.97^{Aa}	3.1 ± 1.09^{Ab}	3.98 ± 0.95^{Aa}	4.1 ± 0.95^{ABa}
Odor	24	3.92 ± 1.04^{Aa}	4.06 ± 1.26^{Aa}	3.82 ± 0.91^{Aa}	4.0 ± 0.72^{Aa}
	48	3.76 ± 0.91^{Aa}	3.84 ± 1.07^{Aa}	3.86 ± 0.85^{Aa}	4.16 ± 0.71^{Aa}
	72	3.56 ± 1.09^{Aab}	3.12 ± 1.20^{Bb}	3.86 ± 1.05^{Aa}	4.06 ± 0.91^{Aa}
General perception	24	3.66 ± 0.96^{Aa}	3.72 ± 1.10^{Aa}	3.72 ± 0.85^{Aa}	4.0 ± 0.75^{Aa}
	48	3.52 ± 0.86^{Ab}	3.4 ± 0.90^{Ab}	3.72 ± 0.88^{Ab}	4.58 ± 0.57^{Ba}
	72	3.4 ± 0.96^{Ab}	2.84 ± 0.86^{Bc}	3.74 ± 0.80^{Aab}	4.08 ± 0.82^{Aa}

[a–c] Different superscript lowercase letters, within a column, denote significant differences ($p<0.05$) between the values obtained for the different treatments according to Duncan test.
[A–B]Different superscripts capital letters, with a row, denote significant differences ($p<0.05$) between values obtained for different days of storage for each treatment according to Duncan test.

However, the increased antimicrobial activity caused by the mixture of these essential oils could be partially explained considering the different compounds found for each essential oil individually. Generally, essential oils having strongest antibacterial activity contain high amounts of carvacrol and/or thymol, such as *O. vulgare*. Regarding that hydroxyl groups enhance the antibacterial properties of essential oils, 1,8-cineole, which was the most prevalent compound in *R. officinalis*, may have also contributed to the establishment of the fast and steady antimicrobial effect achieved with the combination of the oils (Elgayyar, Draughon, Golden, & Mount, 2000). Camphor, one of the main components of *R. officinallis*, possesses oxygen functions in its structure and these functions are known to increase the antimicrobial properties of terpenoids (Naigre, Kalck, Roques, Roux, & Michel, 1996).

Synergy is not only influenced for major compounds of essential oils, because minor components may have a more critical role to this effect than the main components mixed (Gutierrez et al., 2009). Hydrocarbons (such as α-pinene, camphene, myrcene, α-terpinene and *p*-cymene), which have very weak antibacterial activity, found in *R. officinalis* in amounts higher than 1 g/100 g appear to swell bacterial cell to greater extent than carvacrol does, so these compounds enables carvacrol to be more easily transported into the cell (Gutierrez et al., 2008). Dorman and Deans (2000) state that as antimicrobial activity depends not only on chemical composition, but also on the lipophilic properties and the potency of functional groups or aqueous solubility, the mixture of compounds with different biochemical properties may enhance the efficacy of essential oils.

Regarding that the sensory shelf life of foods is determined as the time required for a sensory attribute to reach a certain intensity this study assessed the effect of the essential oils of *O. vulgare* and *R. officinalis* alone (MIC) and in mixture (¼ MIC + ¼ MIC) on the sensory characteristics of minimally processed leafy vegetables over refrigerated storage. Although the essential oils when tested alone were effective in inhibiting the microbial growth in vegetables-based broth and in food matrices, their use to sanitize vegetables caused undesirable effects on sensory attribute mainly after 72 h of storage. Otherwise, the vegetables sanitized with mixture of the essential oils at sub-inhibitory concentrations showed a further acceptability of the most sensory attributes after 72 h of storage.

The results presented in this study showed a synergistic effect of the essential oils of *O. vulgare* and *R. officinalis* on the base of FIC index, kill-time assay and application in fresh leafy vegetables. These essential oils combined at sub-inhibitory concentrations were effective in inhibiting the growth and survival of pathogenic and spoilage microorganisms associated to minimally processed vegetables, although the underlying mode of action remains to be explored in the future. Sensory evaluation suggested that application of the essential oils in mixture at sub-inhibitory concentrations as sanitizer in vegetables would be acceptable to consumers, mainly when regarded a more extended storage time. Our findings reinforced that the mixtures of essential oils with different chemical composition at sufficient low concentration could arise as an alternative to replace synthetic sanitizers classically applied in vegetables, and to reach the balance between the demand for the microbial safety and organoleptic acceptability.

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