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Enhanced antibacterial effectiveness of essential oils vapors in low pressure environment

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ABSTRACT

Due to their antimicrobial activity, essential oils (EOs) have potential to alternate conventional food preservatives. Relatively high doses of EOs necessary for microbial growth inhibition indicate that they should be used in combination with other preservation techniques rather than alone. Therefore, new combinations of preservative methods with EOs are still investigated. In our study, oregano, clove, cinnamon, and lemongrass EO vapors were tested *in vitro* in atmospheric and low pressure against *Escherichia coli* and *Salmonella enteritidis* at different times of treatment ranging from 5 min to 4 h. Combination of EO-low pressure shortened up to 48 times the time necessary for total inhibition of microorganism growth compared to the same treatment in atmospheric pressure. Minimal inhibitory times of EOs vapors ranged mostly from 15 to 60 min in low pressure and were equal to or more than 4 h in atmospheric pressure. Possible decrease of MICs of EOs in low pressure was also investigated. Microorganisms demonstrated increased susceptibility to oregano, lemongrass and cinnamon EOs in low pressure e.g. the MIC of cinnamon vapors for *S. enteritidis* decreased from 512 μL/L to 128 μL/L.

1. Introduction

Essential oils had been extensively used e.g. as food preservatives from ancient times; unluckily, their important role in human life took a back seat with the invention of the new, more precise and predictable, semi-synthetic and synthetic products. However, massive use of these chemicals during the last two centuries resulted in their decreased effectiveness against undesired microorganisms. Moreover, it was reported that some of those substances can have severe side effects on human health (Parke & Lewis, 1992). Those facts and also the new wave of green consumerism brought EOs back to the scope of scientists, food and feed producers, and health industry as well.

EOs antimicrobial, virucidal, antiparasitic and insecticidal effects known from traditional applications have been proven and reviewed by many authors (Bakkali, Averbeck, Averbeck, & Waomar, 2008; Burt, 2004; Lang & Buchbauer, 2012; Tajkarimi, Ibrahim, & Cliver, 2010); furthermore, several other effects, such as antioxidant, anticancer and e.g. anti-nociceptive activity have been demonstrated (Adorjan & Buchbauer, 2010; Edris, 2007).

In vitro antimicrobial activity is probably one of the most often tested characteristic of EOs. Usually, EOs are tested in their liquid phase, but since the last decade, growing interest in their antimicrobial activity in vapor phase can be observed (Tyagi, Malik, Gottardi & Guerzoni, 2012). This method takes the advantage of the natural volatility of EOs, eliminates some of the disadvantages of tests in liquid phase and provides similar or even better results at the same time (Laird & Phillips, 2012). Thanks to that, EOs vapors are understood as substances with high potential in medicine or food industry.

EOs, especially those containing thymol, carvacrol, and cinnamaldehyde (Kloucek et al., 2012), have shown strong antimicrobial activity against common pathogens causing diarrhoeal diseases (*Salmonella enteritidis*, *Escherichia coli*, *Listeria monocytogenes* etc.). Depending on the method, microorganism, and EO, the minimal inhibitory concentrations (MICs) can be as low as 3.9 μg/L (Bakkali et al., 2008).

Experiments performed with the application of EOs against different pathogens on meat, fruit and vegetables, milk and other food products (Tajkarimi et al., 2010; Tiwari et al., 2009) demonstrated in many cases promising results. However, it was usually necessary to increase the EOs' concentration up to 10 times (Burt, 2004) to ensure the same effect as *in vitro*. Unfortunately, such concentrations usually have negative effect on organoleptic properties of tested product; consequently, it makes further use of EOs

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in food industry a bit challenging. On the other hand, it was reported that effective concentrations of EOs can be lowered when EO is applied simultaneously with other commonly used or novel preservative techniques as modified atmosphere, mild heat, high hydrostatic pressure, irradiation, pH reduction, negative air ions, CO_2 – thus when the principle of hurdle technology is applied (Burt, 2004; Lopez, Sanchez, Batlle, & Nerin, 2005; Tyagi et al., 2012).

One of the physical treatment which can support the EOs' effectiveness is vacuum. It is widely used for packaging of different foodstuffs to control the growth of aerobic microflora on food, to prevent fast oxidative changes in products and thus to prolong their shelf life.

Several studies reported reduction of pathogenic microflora in food when the combination of EO and vacuum was employed (Goni et al., 2009; Gorris, Dewitte, & Smid, 1994; Sanchez-Escalante, Djenane, Torrescano, Beltran, & Roncales, 2003). The effect of this combination on microorganism was usually investigated after several hours or days of treatment, which is not suitable for all food products, especially for minimally processed food like vegetable or fruit, because plant tissues can be relatively sensitive to cell damage caused by EO (Bakkali et al., 2008; Tajkarimi et al., 2010). On the other hand, the necessity of preservation of such products can be demonstrated by increasing number of outbreaks of gastroenteritic diseases caused by minimally processed foods (EFSA, 2013).

Thus the aims of our research were to find i) whether the EO vapors in combination with low pressure can shorten the time of treatment necessary to inhibit pathogenic microorganisms compared to the same treatment in atmospheric pressure; ii) whether there is a difference between minimal inhibitory concentrations (MIC) of EOs in atmospheric and low pressure.

2. Materials and methods

2.1. Tested essential oils

Commercially produced EOs of **oregano** (64.5% carvacrol, 5.2% p-cymene and 2.9% thymol), **clove** (82.3% eugenol, 14.4% β-caryophyllene), **lemongrass** (45.34% citral, 33.5% verbenol, 4.0% nerol, 3.3% neryl acetate), **cinnamon** (73.1% cinamaldehyde, 5.0% limonene, 5.0% linalool, 3.7% cinamyl acetate, 3.5% eugenole) were purchased from commercial vendor (Biomedica s.r.o., CZ), their chemical composition was analysed by GS/MS and GC-FID as described elsewhere (Kloucek et al., 2012).

2.2. Tested microorganisms

Two bacterial strains – *S. enteritidis* ATCC 13076 and *E. coli* ATCC 25922 – were purchased from Oxoid (Brno, CZ). Bacterial inocula in concentration 1.5×10^8 (0.5 McFarland) were prepared into Mueller Hinton Broth (MHB) from 24 h old cultures cultivated in MHB at 37 °C.

2.3. In vitro time kill assay in atmospheric and low pressure

2.3.1. Determination of minimal inhibitory concentration (MIC) of essential oils

Firstly, EOs were tested for their minimal inhibitory concentration (MIC) which was determined in atmospheric pressure (101.3 kPa) by the adjusted disc volatilization method (described below – part 2.3.2) for each EO and microorganism separately. MIC was defined as minimal concentration of EOs which completely inhibits visual growth of microorganisms after 24 h of their exposure to EOs. The highest tested concentration was 512 μL/L. For the following tests with vacuum and different treatment duration, these MICs were used.

2.3.2. Disc volatilization method

In vitro tests in atmospheric (101.3 kPa) and low pressure (1.7 kPa) were performed by adjusted disc volatilization method. Petri dishes (6 mm diameter) containing 5 ml agar and 20 ml of air were inoculated with 20 μL of inocula which was than evenly distributed on agar surface. EO diluted in 150 μL of ethyl acetate was poured on filter paper of the same diameter as Petri dish lid. Paper with EO was inserted into the Petri dish after evaporation of ethyl acetate. Seeded agar plate was closed with lid containing the filter paper and stored bottom-up in atmospheric pressure or was placed in the desiccator where the pressure was lowered by oil vacuum pump to 1.7 kPa. Microorganisms were exposed to the EOs vapors for 5, 15, 30, 60, and 120 and minutes in atmospheric and low pressure. After that, filter paper with EO was removed from the Petri dish lid. Growth of bacteria was evaluated after cultivation of treated Petri dishes in 37 °C for 24 h. Each EO was tested in its MIC against both microorganism in all above mentioned times. All tests were done in triplicate. As a control, filter paper with ethyl acetate was used for treatment of inoculated Petri dishes.

3. Results and discussion

3.1. Determination of EOs minimal inhibitory concentrations in atmospheric pressure

MIC of EOs vapors ranged between 64 and 512 μL/L of air (Table 1). The most effective was oregano EO followed by cinnamon and clove oil. Lemongrass vapors did not inhibit the growth of any bacteria even in the highest tested concentration (512 μL/L). *S. enteritidis* was in general less susceptible to tested EOs compared to *E. coli* as only in one case its MIC was lower than 512 μL/L.

MICs of EOs found for *S. enteritidis* comply with our previously reported results (Kloucek et al., 2012). The concentrations were lower when compared with Du et al. (2009; 2008) who reported *in vitro* antimicrobial activity of cinnamon, clove and oregano vapors incorporated in edible films. In those tests *S. enteritidis* was totally inhibited only by oregano EO in 1.5 and 3% w/w concentration while clove and cinnamon demonstrated only partial inhibition of *S. enteritidis* in 3% w/w concentration. On the other hand, study investigating antimicrobial activity of carvacrol vapors against *S. enteritidis* (Burt, Fledderman, Haagsman, van Knapen, & Veldhuizen, 2007) showed that vapors released from 50 μL (approx. corresponds to 1000 μL/L) of carvacrol can significantly reduce the growth of *S. enteritidis*. Further comparable results include different *Salmonella* strains e.g. *Salmonella choleraesuls*

Table 1
Minimal inhibitory times of EOs vapors in atmospheric and subatmospheric pressure.

Essential oil	Microorganism	MIC [μL/L]	MIT [min]*		Time reduction factor
			Pressure		Low/atmosph. pressure
			Low	Atmospheric	
Cinnamon	*S. enteritidis*	512	15	30	2
	E. coli	128	15	240	16
Clove	*S. enteritidis*	512	60	240	4
	E. coli	256	240	1440	6
Lemongrass	*S. enteritidis*	>512	–	–	–
	E. coli	>512	–	–	–
Oregano	*S. enteritidis*	64	15	240	16
	E. coli	64	30	1440	48

*MIT = minimal inhibitory time; MIC = minimal inhibitory concentration determined for 24 h treatment in atmospheric pressure; '–' = not tested; atmospheric pressure = 101.3 kPa, low pressure = 1.7 kPa.