

In vitro* combined effect of oregano essential oil and caprylic acid against *Salmonella* serovars, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Listeria monocytogenes

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Abstract

In vitro inhibition of foodborne pathogens via action of natural antimicrobials – caprylic acid (CA) and essential oil from *Origanum vulgare* L. (OEO) with high carvacrol content (72%) was evaluated. For 15 *Salmonella* strains the minimum inhibitory concentration (MIC) determined by broth microdilution method ranged between 3.67–4.33 $\mu\text{l}\cdot\text{ml}^{-1}$ for CA and between 0.48–0.57 $\mu\text{l}\cdot\text{ml}^{-1}$ for OEO. For 7 *Listeria monocytogenes* strains the MIC ranged between 2.17–2.83 $\mu\text{l}\cdot\text{ml}^{-1}$ for CA and between 0.52–0.58 $\mu\text{l}\cdot\text{ml}^{-1}$ for OEO. Type strains of *Escherichia coli* O157:H7 and *Staphylococcus aureus* were tested, too, with MIC of CA 3.60 $\mu\text{l}\cdot\text{ml}^{-1}$ and 3.20 $\mu\text{l}\cdot\text{ml}^{-1}$ and MIC of OEO 0.51 $\mu\text{l}\cdot\text{ml}^{-1}$ and 0.48 $\mu\text{l}\cdot\text{ml}^{-1}$, respectively. Furthermore, it was found that the MIC of CA can be decreased by even low addition (0.05%) of citric or acetic acid and to a lesser extent by lactic acid, whereas the MICs of OEO did not notably decrease. Combined application of CA and OEO determined by FIC index led only to an additive effect ($0.5 \leq \text{FIC} \leq 1$). Combination of natural additives OEO, CA and eventually acetic or citric acid seems to have the potential to be an effective mixture for inhibition of foodborne pathogens, predominantly *Salmonella* spp. and *L. monocytogenes*, even in only slightly acidic food.

MIC, FIC, natural antimicrobials, *Origanum vulgare*, octanoic acid, foodborne pathogens

In recent years many researchers have focused on natural antimicrobials as an alternative to synthetic food additives, which are not regarded well by consumers. Several studies have proven the essential oil from *Origanum vulgare* L. to be very effective or even the most effective essential oil tested for inhibition of gram-negative bacteria such as salmonellae (Friedman et al. 2002; Nevas et al. 2004; Penalver et al. 2005), which are generally regarded as less sensitive to essential oils. Membrane disintegration seems to be the primary inactivation mechanism of oregano and thyme essential oils (Burt 2004).

Caprylic acid is a saturated medium-chain fatty acid (C_8) with antibacterial and antifungal activity found naturally in coconut oil, bovine and human milk (Jensen et al. 1990). It has been suggested that the antimicrobial activity of medium-chain fatty acids is targeted on cell membrane, leading to disruption of electron transport chain and disruption of oxidative phosphorylation (Desbois and Smith 2010). In a study with C_2 – C_{18} fatty acids caprylic acid was the most effective against *Salmonella* serovars and *E. coli* (Skřivanová et al. 2006).

The application of both essential oils and fatty acids such as caprylic acid in food preservation is limited since generally greater concentrations of these highly aromatic compounds are required to achieve the same effect in food matrix as *in vitro* (Gutierrez et al. 2009; Desbois and Smith 2010). Finding effective combinations of antimicrobials could enable to lower the concentrations of the individual substances to sensory acceptable level.

To our knowledge, the effect of the combined application of medium-chain fatty acids and phenolic compounds against foodborne pathogens has not yet been reported. The aim

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of this study was to evaluate the combined effect of oregano essential oil and caprylic acid and their mixture with other organic acids on inhibition of four foodborne pathogens, *Salmonella enterica*, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Listeria monocytogenes*.

Materials and Methods

Oregano essential oil and organic acids

The oregano essential oil (OEO) from *Origanum vulgare* L. originating from Spain was purchased from Nobilis Tilia (Czech Republic). The major components determined by gas chromatography–mass spectrometry (as reported by producer) were carvacrol (72%), *p*-cymen (7.6%) and γ -terpinen (5.7%). Other chemicals used were caprylic acid (CA, $\geq 98\%$, Sigma-Aldrich, USA) and L-lactic acid (80%, purum), acetic acid (99.8%, p.a.) and citric acid (anhydrous, p.a.) purchased from Lach-Ner, Czech Republic.

Stock solutions (48%) of oregano essential oil and caprylic acid were prepared in ethanol and further diluted with Tryptic Soy Broth (TSB, Oxoid, UK). The other acids were diluted in distilled water to form 10% solutions (w/v) and further diluted with TSB.

Bacterial strains

The bacteria used in this study were *Salmonella enteritidis* ATCC 13076, *Escherichia coli* O157:H7 ATCC 700728, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 13932 and six *Listeria monocytogenes* strains 1/2a, 1/2b and 1/2c isolated from minced meat, heat-treated and non-heat-treated meat products. Fourteen *Salmonella* strains including Enteritidis PT1b, PT4, PT4b, PT8, PT13, PT21, Typhimurium DT104 and DT120 and serotype Montevideo isolated from meat, meat products and environmental swabs from meat processing plants were kindly provided by National Public Health Institute, Czech Republic. All strains were kept frozen at $-80\text{ }^{\circ}\text{C}$ in TSB with 20% glycerol. Before using the cultures were twice sub-cultivated in TSB at $37\text{ }^{\circ}\text{C}$ for 24 h and then diluted in TSB to approximately 3×10^6 cells·ml⁻¹ using the McFarland turbidity scale.

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of OEO and CA were determined using 96-well microtitre plates (Gamma group, Czech Republic). Each well contained 100 μl of the OEO solution (final concentration 0.1–1 $\mu\text{l}\cdot\text{ml}^{-1}$) or CA solution (final concentration 1–10 $\mu\text{l}\cdot\text{ml}^{-1}$) and 50 μl of bacterial suspension. Negative controls and positive controls in TSB with ethanol were included. The plates were incubated at $37\text{ }^{\circ}\text{C}$ for 24 h and the MIC was determined as the lowest concentration required to prevent visible growth. Experiments were performed in duplicate and replicated 3 \times .

Determining the effect of organic acids on MICs

The effect of organic acids on microbial inhibition by OEO and CA was determined by the same method. Each well contained 50 μl of OEO or CA solution (final concentrations as previously mentioned), 50 μl of lactic, acetic or citric acid (final concentration 0.05%) and 50 μl of bacterial suspension. Experiments were performed in duplicate and replicated 3 \times .

Checkerboard method

The checkerboard method was performed using 96-well microtitre plates to obtain the fractional inhibitory concentration (FIC) index. Each well contained 50 μl of OEO solution (final concentration 0.0125–0.6 $\mu\text{l}\cdot\text{ml}^{-1}$), 50 μl of CA solution (final concentration 0.125–6 $\mu\text{l}\cdot\text{ml}^{-1}$) and 50 μl of bacterial suspension. Negative and positive controls were included and the plates were incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. The FIC indices were calculated according to Gutierrez et al. (2009) as $\text{FIC}_{\text{OEO}} + \text{FIC}_{\text{CA}}$, where $\text{FIC}_{\text{OEO}} = \text{MIC}_{\text{OEO}}$ in combination/ MIC_{OEO} alone and $\text{FIC}_{\text{CA}} = \text{MIC}_{\text{CA}}$ in combination/ MIC_{CA} alone. The results were interpreted as synergy ($\text{FIC} < 0.5$), addition ($0.5 \leq \text{FIC} \leq 1$), indifference ($1 < \text{FIC} \leq 2$) or antagonism ($\text{FIC} > 2$). Experiments were performed in triplicate.

Statistical evaluation

Kruskal-Wallis ANOVA was performed by StatPlus 2008 Professional v. 5.2.5.0 software. A level of $P < 0.05$ was considered significant. FIC indices were not subjected to analysis since not enough data were obtained for statistical evaluation.

Results

The MICs of OEO and CA

Minimum inhibitory concentrations of CA against *Salmonella* and *Listeria monocytogenes* wild types ranged between 3.67–4.33 $\mu\text{l}\cdot\text{ml}^{-1}$ and 2.17–2.83 $\mu\text{l}\cdot\text{ml}^{-1}$, respectively, whereas the MICs of OEO ranged between 0.48–0.57 $\mu\text{l}\cdot\text{ml}^{-1}$ and 0.55–0.58 $\mu\text{l}\cdot\text{ml}^{-1}$, respectively. The MICs of type strains are listed in Table 1. No significant difference was observed in MIC of CA and OEO among *Salmonella* serovars and the same held true for *Listeria*

Table 1. Sensitivity of bacterial type strains to caprylic acid, oregano essential oil and their combination

Strain	Type strain number	MIC of CA ($\mu\text{l}\cdot\text{ml}^{-1}$) (mean \pm SD)	MIC of OEO ($\mu\text{l}\cdot\text{ml}^{-1}$) (mean \pm SD)	FIC (mean \pm SD)
<i>Salmonella</i> Enteritidis	ATCC 13076	4.33 \pm 0.52 ^a	0.50 \pm 0.09	0.84 \pm 0.00
<i>Escherichia coli</i> O157:H7	ATCC 700728	3.60 \pm 0.52 ^{ab}	0.51 \pm 0.09	0.94 \pm 0.11
<i>Staphylococcus aureus</i>	ATCC 25923	3.20 \pm 0.75 ^b	0.48 \pm 0.08	0.91 \pm 0.00
<i>Listeria monocytogenes</i>	ATCC 13932	2.36 \pm 0.67 ^c	0.52 \pm 0.08	0.82 \pm 0.18

MIC - minimum inhibitory concentration, FIC - fractional inhibitory concentration, CA - caprylic acid, OEO - oregano essential oil, a,b,c - significant difference ($P < 0.05$)

monocytogenes strains. *L. monocytogenes* strains were the most sensitive against CA with average MIC of only $2.36 \pm 0.22 \mu\text{l}\cdot\text{ml}^{-1}$, followed by *S. aureus* and *E. coli*, while the salmonellae were the most resistant ($4.22 \pm 0.18 \mu\text{l}\cdot\text{ml}^{-1}$). There was no significant difference in MICs of OEO among type strains; however, *Listeria monocytogenes* strains seemed to be the most resistant with average MIC of $0.56 \pm 0.02 \mu\text{l}\cdot\text{ml}^{-1}$.

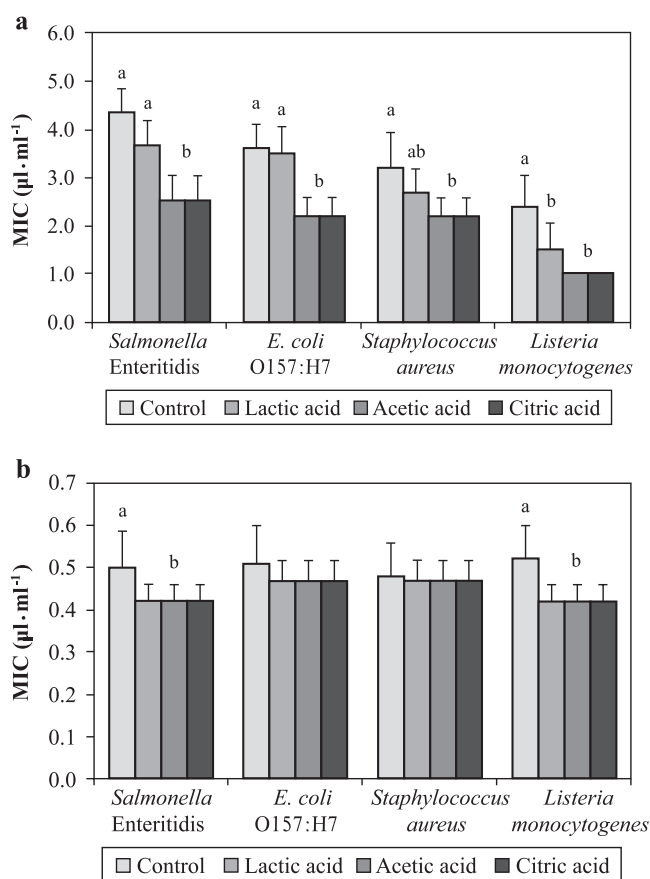


Fig. 1. Effect of acids (0.05% w/v) on minimum inhibitory concentration (MIC) of caprylic acid (a) and oregano essential oil (b) for bacterial type strains with statistical differences between controls and acids marked

The effect of organic acids on MICs of OEO and CA

The effect of organic acids was tested only on bacterial type strains. In presence of short-chain organic acids (lactic, acetic and citric), the MICs of CA were lowered considerably (Fig. 1a), especially of *Listeria monocytogenes* and to a lesser extent of *Salmonella* Enteritidis. The effect of acetic and citric acid on MICs of CA was much more pronounced in comparison with lactic acid. The MICs of OEO were less affected (Fig. 1b) as only MICs of OEO against *L. monocytogenes* ($P = 0.018$) and *Salmonella* Enteritidis ($P = 0.043$) were lowered significantly in presence of organic acids.

The combined effect of OEO and CA

Combined effects of the antimicrobials in question were evaluated by means of FIC indices listed in Table 1. The FIC indices for type strains varied between 0.82 and 0.94. These values (between 0.5 and 1.0) can be interpreted as an additive effect.

Discussion

Despite no significant differences in MIC OEO between bacterial type strains, the strains of *L. monocytogenes* seemed to be the most resistant followed by *Salmonella enterica* strains. Together with *S. aureus* type strain being the most sensitive, no general conclusions about different resistance of gram-positive and gram-negative bacteria can be made in our case. Higher sensitivity of gram-positive bacteria (e.g. *L. monocytogenes*, *S. aureus*) compared to gram-negative bacteria (*S. Typhimurium*, nonpathogenic *E. coli*) to OEO was reported by Nevas et al. (2004). However, in a study of Di Pasqua et al. (2005), the MIC increased as followed: *S. Typhimurium*, *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*, suggesting the opposite. Friedman et al. (2002) observed similar sensitivity of *E. coli* O157:H7 and *Salmonella* Hadar strains to OEO, whereas *L. monocytogenes* was more resistant.

The choice of strains can play an important role in these comparisons since the MICs often vary considerably between strains. Although no major differences were noted in our case, in a study of Penalver et al. (2005) the MIC of OEO ranged from 2.5 $\mu\text{l}\cdot\text{ml}^{-1}$ for *Salmonella* Enteritidis up to 10 $\mu\text{l}\cdot\text{ml}^{-1}$ for serovars Typhimurium and Essen. The same results were obtained for caprylic acid. No major differences among various *Salmonella* and *L. monocytogenes* strains were found in this study, although e.g. Boyen et al. (2008) measured different MICs of CA against 54 porcine *Salmonella* Typhimurium strains. In our study, greater sensitivity of gram-positive bacteria to caprylic acid compared to gram-negative bacteria was observed. *L. monocytogenes* was the most sensitive followed by *S. aureus*, *E. coli* O157:H7 and *Salmonella* Enteritidis. Similarly, Nobmann et al. (2009) observed greater sensitivity of *L. monocytogenes* (MIC 5 mM) followed by *E. coli* O157:H7 (MIC 10 mM) and *Salmonella* Typhimurium (MIC > 20 mM).

Simultaneous application of CA and OEO tested on bacterial type strains led to an additive effect, with FIC ranging between 0.82 and 0.94 (Tab. 1). The lowest FIC index was noted for *L. monocytogenes* and *Salmonella* Enteritidis. Although no synergy was detected between OEO and CA, the additive effect still enables to obtain the same antimicrobial effect as that of OEO or CA alone using lower concentrations of both of the substances simultaneously. The effective concentration can be very important for use in food since both CA and OEO have a distinctive odour and the efficiency of essential oils is generally lowered in foodstuffs such as meat, requiring higher concentrations that are often sensory unacceptable (de Souza et al. 2009; de Oliveira et al. 2010).

As a part of this study we investigated whether the effective concentrations could be further lowered by addition of other organic acids in subinhibitory concentrations. At lower pH the essential oils are more hydrophobic and can more easily dissolve in lipids of bacterial cell membrane (Juven et al. 1994). Several authors reported synergy or

additive effect of OEO (or its main component carvacrol) and lactic, acetic or citric acid against foodborne pathogens (Dimitrijevic et al. 2007; Zhou et al. 2007; de Souza et al. 2009; de Oliveira et al. 2010). In our study the presence of lactic, acetic or citric acid at concentration of either 0.05% (Fig. 1b) or 0.1% (unpublished data) did not lead to a significant reduction of MIC of OEO with the exception of *L. monocytogenes* ($P = 0.018$) and *S. Typhimurium* ($P = 0.043$). The presence of buffering substances (dipotassium hydrogen phosphate) in the cultivating medium and consequently minimal drop of pH (6.8 - 7.3; unpublished data) could explain this failure.

The effect of addition of other organic acids on MIC of CA was more pronounced. At low pH, more of the organic acid will be in the undissociated form which can diffuse across the cell membrane (Ricke 2003). The enhanced effectiveness of CA at low pH is well known and has been reported against both *Salmonella* spp. and *Listeria monocytogenes* (Kinderlerer and Lund 1992; Boyen et al. 2008). *L. monocytogenes* was the most affected bacterial strain in our study, followed by *Salmonella* Enteritidis and *E. coli* O157:H7. Since *L. monocytogenes* and *Salmonella* Enteritidis were the most affected strains in lower pH regarding both MIC of CA and OEO, individual sensitivity of these strains to acidic conditions can play a role.

The results of this study demonstrate the effectiveness of oregano essential oil in combination with caprylic acid against foodborne pathogens *in vitro*. Further research is needed to evaluate the effectiveness in food substrates and the sensory acceptability of the effective mixture.

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